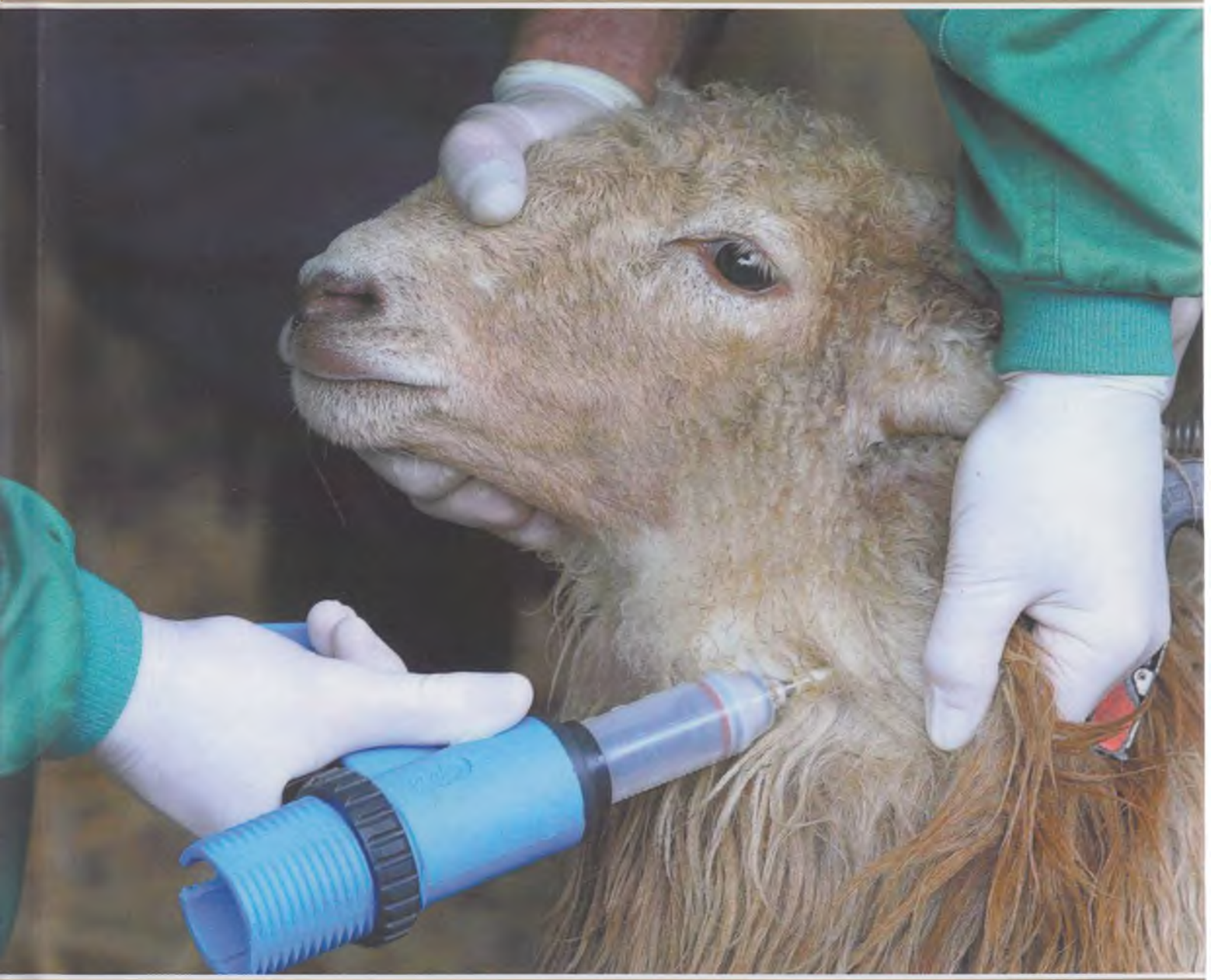
Veterinary Medicine

Assessment, Diagnosis and Treatment of Animal Diseases

Kevin McLeod



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Preface

This book has been an outcome of determined endeavour from a group of educationists in the field. The primary objective was to involve a broad spectrum of professionals from diverse cultural background involved in the field for developing new researches. The book not only targets students but also scholars pursuing higher research for further enhancement of the theoretical and practical applications of the subject.

Veterinary medicine is the discipline that focuses on the prevention, diagnosis and treatment of animal disorders, injuries and diseases. It deals with all animal species, both domestic and wild. Professionals in this field are known as vets or veterinary surgeons. They might be assisted by veterinary technicians and nurses. The diagnosis of diseases in animals is primarily based on clinical signs. This is due to the fact that animals are unable to vocalize their symptoms. In certain cases, the results of diagnostic tests such as radiography, MRI and CT scans, are also consulted. Some of the specialties within veterinary medicine are anaesthesiology, immunology, oncology and radiology. This book explores all the important aspects of veterinary medicine in the present day scenario. Some of the diverse topics covered in this book address the varied branches that fall under this field. It will help new researchers by foregrounding their knowledge in veterinary medicine.

It was an honour to edit such a profound book and also a challenging task to compile and examine all the relevant data for accuracy and originality. I wish to acknowledge the efforts of the contributors for submitting such brilliant and diverse chapters in the field and for endlessly working for the completion of the book. Last, but not the least; I thank my family for being a constant source of support in all my research endeavours.

Editor

Long-term monitoring of opioid, sedative and anti-inflammatory drugs in horse hair using a selective and sensitive LC-MS/MS procedure

Milena M. Madry[1*], Barbara S. Spycher[2], Jacqueline Kupper[3], Anton Fuerst[4], Markus R. Baumgartner[1], Thomas Kraemer[2] and Hanspeter Naegeli[3*]

Abstract

Background: Compared to blood or urine, drugs can be detected for much longer periods in the long hair of horses. The aim of this study was to establish and validate a highly sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the detection and quantification of frequently prescribed opioids, sedatives and non-steroidal anti-inflammatory agents in the mane and tail hair of horses. Based on an average growth rate of about 2 cm per month, times of administration reported by horse owners or veterinary physicians were related to drug localizations in hair. Hair samples were collected from ten horses that received drug treatments and analyzed in segments of 2, 4 or 6 cm in length. Hair segments were decontaminated, cut into fragments and methanol-extracted under sonication. The extracts were analyzed by LC-MS/MS for 13 commonly used drugs using the validated procedure. Deuterated analogs were included as internal standards.

Results: Analytes were detected in hair samples with a length of up to 70 cm. Fourteen out of 16 hair samples were positive for at least one of the tested drugs. Segmentation allowed for time-resolved monitoring of periods of 1 to 3 months of drug administration. Concentrations in dark hair reached a maximum of 4.0 pg/mg for butorphanol, 6.0 pg/mg for tramadol, 1.4 pg/mg for morphine, 1.8 pg/mg for detomidine, 1.2 pg/mg for acepromazine, 39 pg/mg for flunixin, 5.0 pg/mg for firocoxib, and 3'600 pg/mg for phenylbutazone. Only trace amounts of meloxicam were detected. Drug detection correlated well with the reported period of medical treatment. No analytes were detected in the light-colored mane and tail hair samples from one horse despite preceding administrations of acepromazine and phenylbutazone.

Conclusion: This study describes a sensitive and selective technique suitable for the validated detection and quantification of frequently prescribed veterinary drugs in horse hair. The segmental method can be applied for time-resolved long-term retrospective drug monitoring, for example in prepurchase examinations of horses as drug detection in hair can prove preceding medical treatments.

Keywords: Doping, LC-MS/MS, Pre-purchase examination, Horse hair

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Background

Horse hair analysis has gained increasing interest for the monitoring of veterinary drugs, hormones, nutrients, trace elements and contaminants [1–7]. In contrast to blood and urine, hair provides a long-term historical record of drug exposure that may be useful for applications in sports anti-doping control programs, pre-purchase examination of horses and examinations for insurance purposes [2]. Other advantages of hair analyses include the non-invasive probing as well as drug-stability in hair and easy shipping of samples [8]. The growth of temporary hair of the coat changes with seasons whereas the permanent mane and tail hair grows continuously with relatively constant rates reported in the range from 1.7 to 2.5 cm per month [1, 2, 9, 10]. Therefore, samples of long hair can be segmented to narrow down the corresponding times of drug exposure [2, 11].

So far, detection in horse hair has been demonstrated for morphine [1], clenbuterol [3], anabolic steroids [4, 7, 9], antimicrobial agents [12], diazepam [13], cortisol [14], arsenic [6] and selenium [15]. The aim of this study was twofold. The first goal was to extend the use of mane and tail hair as a matrix for the monitoring of sedative, analgesic and anti-inflammatory drugs that are frequently prescribed to horses. The second goal was to compare closely the analytical findings in hair segments with the corresponding times of drug administration. To that end, a highly sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was established, validated and applied to mane and tail hair samples of 10 horses with a known history of pharmacologic treatments.

Methods

Study design and sample collection

The study included ten horses with documented administration of at least one of the investigated drugs before sampling. All horse owners provided informed consent to participate in the study. Sixteen mane and nine tail hair samples were collected by horse owners or veterinarians. One tail hair sample was collected post-mortem. The sampling method was adapted from guidelines of the Society of Hair Testing (SoHT) [11]. A hair lock was fixed with the hair strands equally aligned using a string and cut precisely close to the skin. The lock was wrapped in aluminum foil with the proximal ends aligned for clear identification. Samples were stored at room temperature under dry conditions and in the dark. Individual data on drug administration, hair type and color of each horse are reported in Table 1.

Table 1 Drug treatment of the horses (relative to time of sample collection) and characteristics of hair samples

Horse	Reported drug (s)	Time before sampling	Hair samples (length, color)
P01	Acepromazine	2 months	Mane (16 cm, light) Tail (28 cm, light)
	Phenylbutazone	3 and 9 months	
P02	Butorphanol	22 months	Tail (70 cm, dark)
	Detomidine	With euthanasia	
	Flunixin	1 day and 22 months	
P03	Flunixin	8 months	Mane (16 cm, dark) Tail (28 cm, dark)
	Ketoprofen	8 months	
P04	Flunixin	9 months	Mane (16 cm, dark) Tail (26 cm, dark)
	Ketoprofen	9 months	
	Phenylbutazone	10 months	
P05	Flunixin	7 months	Mane (16 cm, dark) Tail (28 cm, dark)
P06	Acepromazine	15 months	Tail (68 cm, dark)
	Butorphanol	17 months	
	Phenylbutazone	8 months	
P07	Flunixin	2 and 4 months	Mane (24 cm, dark) Tail (26 cm, dark)
	Phenylbutazone	2 and 4 months	
P08	Butorphanol	6 months	Mane (20 cm, dark)
	Detomidine	6 months	
	Phenylbutazone	10 months	
	Tramadol	6 months	
P09	Flunixin	1 week	Mane (24 cm, white) Tail (24 cm, dark)
	Phenylbutazone	1 week	
P10	Butorphanol	8 months	Tail (44 cm, dark)
	Firocoxib	10 months	
	Meloxicam	10 months	

Analytical standards and chemicals

Morphine, morphine-d_3, tramadol, ^{13}C-tramadol-d_3, buprenorphine-d_4, chlorpromazine-d_3, and phenylbutazone were obtained as solutions from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). Butorphanol and buprenorphine were obtained as solutions from Resea-Chem GmbH (Burgdorf, Switzerland) and Lipomed AG (Arlesheim, Switzerland), respectively. Ketoprofen-d_3 and phenylbutazone-(diphenyl$^{13}C_{12}$) were purchased as powders Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). Detomidine, acepromazine, meloxicam, ketoprofen, chlorpromazine, flunixin and fluphenazine were analytical standards available as powders in the authors' laboratory. All solvents and other chemicals were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). All standards were dissolved in methanol, except for meloxicam that was dissolved in dimethylformamide, to yield stocks of 1 mg/ml. Firocoxib was obtained as an injectable for veterinary use. Chromatography-grade water used for LC-MS/MS analysis was processed by a PURELAB Option-Q system by ELGA LabWater (Labtec Services AG, Villmergen, Switzerland).

Preparation of working solutions

Spiking solutions for calibrators and quality control (QC) samples were prepared in methanol to yield concentrations comparable to those found in hair. An internal standard (IS) solution with the following concentrations was prepared in methanol: morphine-d_3 (15 ng/ml), ^{13}C-tramadol-d_3 (8.0 ng/ml), buprenorphine-d_4 (20 ng/ml), ketoprofen-d_3 (10 ng/ml), chlorpromazine-d_3 (10 ng/ml) and phenylbutazone-(diphenyl$^{13}C_{12}$) (30 ng/ml).

Hair sample preparation

Mane and tail hair samples were cut into 2- or 4-cm segments; one very long hair sample of 70 cm was analyzed in 6-cm segments. For a thorough decontamination according to the laboratory standard procedure for forensic hair analysis, 10 mg of hair were washed once with 5 mL deionized water and twice with 5 mL acetone for 3 min each. After drying at room temperature, hair segments were chopped in snippets with scissors. For extraction, 100 µl IS solution and 1900 µl methanol were added and samples were sonicated for 4 h at 50 °C. After centrifugation for 7.5 min at 10,000 g, 1500 µl of the clear solution was transferred into a vial for evaporation under a stream of nitrogen at 40 °C. For injection into the LC-MS/MS system, the residue was reconstituted in 60 µl 5 mM ammonium formate[a] (pH 3) with 10 % (v/v) methanol.

LC-MS/MS parameters

Analytes were separated on a C18 column (Phenomenex Kinetex 2.6 µm C18 column, 50 × 2.1 mm, Brechbühler AG, Schlieren, Switzerland) using a high-performance LC system (Ultimate 3000 HPLC system, Thermo Fisher Scientific AG, Reinach, Switzerland) and detected by a linear ion trap triple quadrupole mass spectrometer (Applied Biosystems 5500 Q Trap with Analyst software, Sciex, Darmstadt, Germany). The mobile phase consisted of 5 mM ammonium formate buffer (pH 3; eluent A) and methanol containing 5 mM ammonium formate (eluent B). The following gradient was used: 10 to 50 % B from 0 to 3 min, 50 % B from 3 to 10 min, 50 to 90 % B from 10 to 10.5 min and 90 % B from 10.5 to 11.5 min. The flow rate was 0.35 ml/min. The column temperature was 35 °C and injection volumes were 10 µl. The MS instrument was operated in positive electrospray ionization mode. Three specific multiple reaction monitoring (MRM) transitions per substance were analyzed, but for detomidine only two transitions could be recorded (Table 2). Quantification was achieved using the most abundant transition of each precursor to the respective product ion. Criteria for positive identification were the matching retention time, three specific MRM transitions (one served as quantifier and two served as qualifier), and the matching relative ion intensities of MRM transitions (qualifier-to-quantifier) which were determined from calibrators ($n = 10$ each) (Table 2). The tolerance level for percent deviations of relative intensities of MRM transitions was ±30 % [16]. The absence of interfering peaks was verified for all analytes in blank samples and after addition of the IS solution.

Method validation

The method was validated for selectivity, limit of detection (LOD), lower limit of quantification (LLOQ), linearity of calibration, accuracy, intra-day and inter-day precision, and matrix effects based on the Guidelines for Quality Control in Forensic-Toxicological Analyses [17, 18]. All validation parameters were assessed with drug-free hair samples (blank hair) which were obtained from horses that had never received medications. Drug-free hair was spiked with 100 µl IS solution and 100 µl calibrator or QC solution. The calibration line was calculated using a weighted [1/x, x = f (concentration)] linear regression model. Calibration curves with seven calibrators were prepared for eight days. LOD was defined as the lowest concentration with a peak height showing a signal-to-noise ratio of at least 3:1 in the chromatogram, (Table 3). The LLOQ showing a signal-to-noise of at least 10:1 was chosen as the lowest calibrator concentration; LLOQ levels ranged from 0.1 to 5 pg/mg, except for phenylbutazone for which an LLOQ of 25 pg/mg was determined. Accuracy and precision were determined via daily calibration curves over eight days by analyzing duplicate QC samples at low, medium, and high concentrations (Table 3). The accepted intervals for precision were ± 15 % (±20 % at LLOQ) for bias and standard deviation of the nominal concentration. Processed sample stability in the auto-sampler at room temperature was tested by pooling extracts prepared at these low and high concentrations. Six aliquots were injected in the LC-MS/MS system every 2 h over 18 h. Further stability studies were not included as samples were tested immediately after their preparation. Matrix effects were studied with hair samples of different sources as previously proposed [17, 19]: the peak area of analytes in blank hair samples of different horses ($n = 5$) spiked after the extraction was divided by the mean peak area of analytes in neat standard solutions ($n = 5$) at the same concentration level and multiplied by 100. Values below or above 100 indicate ion suppression or ion enhancement resulting in diminished or increased signal intensities.

Results

Method validation

An LC-MS/MS method was developed for the detection and quantification of 13 sedative, analgesic and anti-inflammatory drugs commonly used in horses. A

Table 2 LC-MS/MS parameters

Analyte/internal standard	Precursor ion (m/z)	Product ion (m/z)	Relative ion intensities (mean) ± rSD (%)[a]	RT (min)	DP (V)	EP (V)	CE (V)	CxP (V)
Morphine	286.1	**152.1**	0.7 ± 16	0.6	156	10	81	14
		128.1		0.6	156	10	79	16
		165.1		0.6	156	10	57	16
Tramadol	264.05	**58.0**	0.32 ± 6.2	3.4	46	10	17	10
		58.5		3.4	46	10	33	8
		42.0		3.4	46	10	115	20
Detomidine	187.09	**81.1**	0.23 ± 7.7	3.9	101	10	25	8
		54.1		3.9	101	10	55	8
Butorphanol	328.17	**310.2**	0.15 ± 3.6	4.2	101	10	33	22
		157.0		4.2	101	10	59	12
		131.1		4.2	101	10	65	12
Buprenorphine	468.24	**55.1**	0.05 ± 12	5.1	1	10	101	6
		41.1		5.1	1	10	129	4
		115.1		5.1	1	10	129	6
Acepromazine	327.04	**86.0**	0.99 ± 5.6	5.5	81	10	25	10
		58.0		5.5	81	10	65	8
		222.1		5.5	81	10	53	16
Firocoxib	337.02	**283.1**	0.81 ± 7.1	5.6	101	10	13	16
		130.1		5.6	101	10	41	12
		237.0		5.6	101	10	23	20
Meloxicam	351.96	**115.0**	0.37 ± 7.0	6.3	126	10	25	10
		141.0		6.3	126	10	27	12
		73.0		6.3	126	10	75	12
Chlorpromazine	319.26	**58.1**	0.89 ± 4.2	6.7	86	10	67	12
		86.1		6.7	86	10	25	8
		214.0		6.7	86	10	57	20
Ketoprofen	255.08	**77.0**	0.93 ± 6.7	6.7	131	10	61	12
		104.9		6.7	131	10	33	12
		51.1		6.7	131	10	99	8
Flunixin	297.03	**278.9**	0.38 ± 3.1	7.3	111	10	33	24
		264.0		7.3	111	10	47	22
		236.1		7.3	111	10	57	16
Fluphenazine	438.22	**171.0**	0.71 ± 2.8	9.3	56	10	37	20
		143.1		9.3	56	10	37	18
		70.0		9.3	56	10	67	10
Phenylbutazone	309.15	**77.0**	0.51 ± 9.6	9.5	216	10	75	12
		160.1		9.5	216	10	29	14
		120.0		9.5	216	10	27	10
Morphine-d_3	289.02	**201.0**		0.6	156	10	35	16
		152.1		0.6	156	10	75	18
^{13}C-Tramadol-d_3	268.07	**58.0**		3.4	46	10	49	14
		42.1		3.4	46	10	113	20

Table 2 LC-MS/MS parameters *(Continued)*

Buprenorphine-d_4	472.26	**59.0**	5.1	1	10	107	6
		400.1	5.1	1	10	53	2
Chlorpromazine-d_3	322.04	**89.1**	6.7	51	10	25	10
		61.1	6.7	51	10	59	8
Ketoprofen-d_3	258.09	**212.1**	6.7	121	10	19	14
		105.0	6.7	121	10	31	10
$^{13}C_2$-Phenylbutazone	321.11	**166.2**	9.5	76	10	29	14
		55.1	9.5	76	10	129	8

MRM (multiple reaction monitoring mode) transitions with precursor and product ions, relative ion intensities of MRM transitions (qualifier-to-quantifier); *RT* retention time, *DP* declustering potential, *EP* entrance potential, *CE* collision energy, *CxP* collision cell exit potential. MRM transitions used as quantifiers are given in bold

[a]determined from calibrators (n = 10, per analyte)

representative chromatogram including all analytes is given in Fig. 1. The method, validated according to the Guidelines for Quality Control in Forensic-Toxicological Analyses [17, 18], was selective and very sensitive for all analytes. The detection of buprenorphine, butorphanol, detomidine, firocoxib, flunixin, ketoprofen, meloxicam, morphine and phenylbutazone fulfilled all validation criteria (Table 4). For chlorpromazine and tramadol, matrix effects exceeded the limits of ± 25 % standard deviation, but this was compensated by the inclusion of deuterated standards. The detection of acepromazine and fluphenazine displayed deviations in accuracy as well as precision, and variable matrix effects were observed. However, inclusion of labeled analogs of the two compounds allowed for comparisons between samples from the same horse. For all other drugs, the method was successfully validated and proven to be suitable for the comparison of hair samples from different horses.

Detection of opioids

Butorphanol was detected in the hair of four out of five horses having received the drug. The concentrations ranged from below LLOQ to 4.0 pg/mg. In horse P02, repeated administrations of butorphanol were reported 22 months before sample collection and the highest concentration (4.0 pg/mg) was detected in a tail hair segment corresponding to a distance from the skin of 36 to 42 cm (Fig. 2a). In horse P08, a single application of butorphanol 6 months before sampling resulted in drug concentrations in tail hair of 0.9–2.3 pg/mg spread across a distance of 6–16 cm from the skin. In many cases, more drugs were found in hair than reported to have been administered. For example, butorphanol was detected in multiple 2-cm segments of the mane and tail hair of horse P07 (peak concentration of 3.6 pg/mg at a distance of 18–20 cm from the skin) and P10 (peak of 0.6 pg/mg at 22–24 cm from the skin), although

Table 3 Analyte concentrations in method validation: for limit of detection (LOD), lower limit of quantification (LLOQ), calibration range and quality control (QC) samples

Analyte	LOD (pg/mg)	LLOQ (pg/mg)	Cal. range (pg/mg)	QC low (pg/mg)	QC medium (pg/mg)	QC high (pg/mg)
Morphine	0.1	0.6	0.6–840	0.585	270	555
Tramadol	0.1	0.5	0.5–100	0.48	30	68
Detomidine	0.1	0.6	0.6–36	0.585	11	24
Butorphanol	0.1	0.5	0.5–50	0.48	15	33
Buprenorphine	1	2	2–800	1.95	225	540
Acepromazine	0.05	0.1	0.1–150	0.098	45	101
Firocoxib	0.5	1	1–740	0.975	225	495
Meloxicam	0.5	2	2–280	1.95	90	188
Ketoprofen	1	5	5–420	4.8	135	278
Chlorpromazine	0.5	1	1–280	0.975	90	189
Flunixin	0.05	0.1	0.1–66	0.098	223	44
Fluphenazine	0.5	1	1–600	0.975	180	405
Phenylbutazone	10	25	25–7000	24	2250	4650

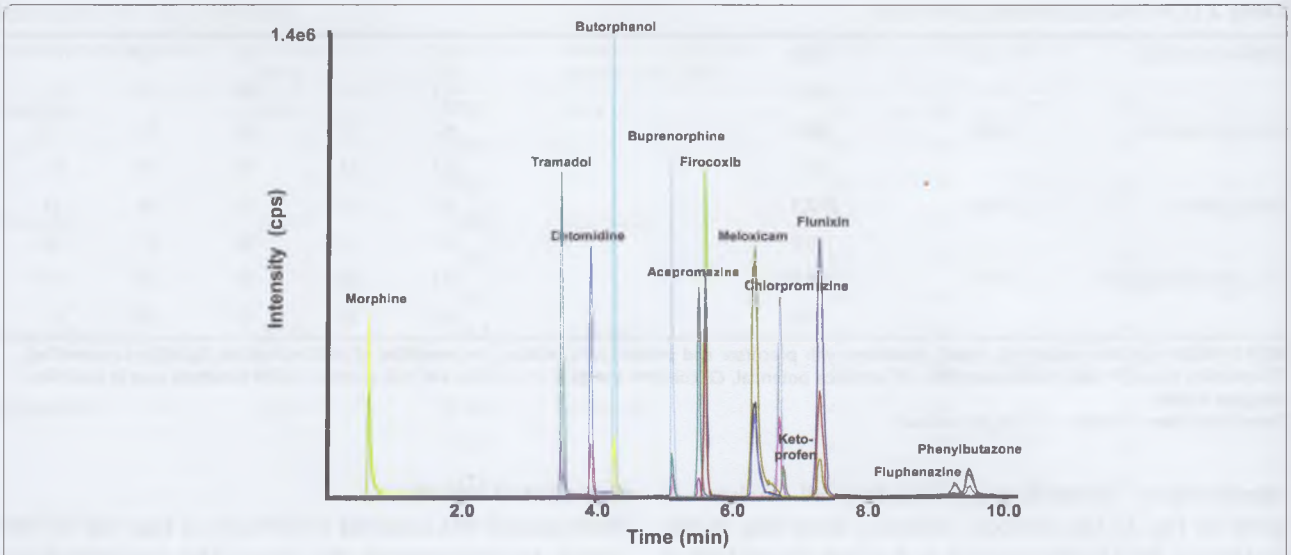

Fig. 1 LC-MS/MS chromatogram of a spiked sample with the respective MRM transitions for 13 investigated drugs

administration of this drug was not reported. However, the presence of butorphanol was not demonstrated in the dark tail hair of horse P06 treated once 17 months before sample collection.

A single dose of tramadol was administered to horse P08 around 6 months before sample collection. This treatment resulted in a distinctive analyte peak (6 pg/mg) in a unique 2-cm segment of tail hair located 8–10 cm from the skin (Fig. 2b). A distinctive peak of the same drug (1.1 pg/mg) was also found in the tail hair of horse P02 but without any documentation of previous tramadol use. Morphine was present in the tail hair sample of horse P02 at concentrations of up to 1.4 pg/mg, although medication with this analgesic drug had not been recorded. There was no report of buprenorphine administration and, accordingly, all samples were negative for this substance.

Detection of sedatives

A single detomidine application was given to horse P02 on the day of euthanasia. This resulted in detection of traces of the drug around or below the LLOQ in all tested mane and tail hair segments, indicating an external contamination by sweat or other biological fluids. Detomidine was also detected in the mane hair of horse P07, with maximum levels (1.8 pg/mg) at a distance of 4–6 cm from the skin, although administration of this drug was not reported. On the other hand, a single dose of detomidine 6 months before sampling could not be detected in the dark mane hair of horse P08. Acepromazine, given to horse P06 around 15–16 months before sampling, was retrieved at concentrations of 0.5–1.2 pg/mg in tail hair segments corresponding to a distance of 8–40 cm from the skin (Fig. 2c). There was no report of chlorpromazine and fluphenazine administration and none of the samples contained these drugs.

Detection of non-steroidal anti-inflammatory agents

All hair samples of the four horses with reported flunixin administration were positive for this common anti-inflammatory agent, the highest detected concentration being 39 pg/mg. A sharp peak of flunixin deposition was observed in the tail hair of horse P03. In this case, drug administrations occurring 8 months before sampling yielded a distinctive accumulation at a distance of 16–18 cm from the skin (Fig. 2d). It should be noted that flunixin concentrations were higher in tail (up to 3.3 pg/mg) than in mane hair segments (below LLOQ). This trend was confirmed by comparing flunixin residues in the hair of horses P07 and P09 (data not shown). Firocoxib was detected in the tail hair of horse P10 at concentrations up to 5.0 pg/mg and at a distance of 12–16 cm from the (Fig. 2e). This residue is due to firocoxib treatments given 10 months before sampling.

Phenylbutazone administration was reported in six horses, but the substance was detected in the hair of only two of these animals. This is a consequence of the relatively high LOD (10 pg/mg) linked to poor ionization of phenylbutazone in the MS system. To our knowledge, incorporation of phenylbutazone into hair has not been described before. However, we were able to detect this drug along the entire tail hair of horse P06 with the highest concentration of 1300 pg/mg at a distance of 52–56 cm from the skin. This localization corresponds to a non-recorded treatment that may have been carried out more than 2 years before sampling. Phenylbutazone was also detected in the mane hair of horse P08 (Fig. 2f). In this case, the

Table 4 Validation parameters: accuracy, intra-day and inter-day precision, matrix effects. N/A, not determined

Analyte	QC sample	Accuracy, bias (%)	Intra-day precision, SD (%)	Inter-day precision, SD (%)	ME, mean ± SD (%)
Morphine	Low	0.6	6.2	8.8	56 ± 6.9
	Medium	3.2	3.5	3.8	N/A
	High	−0.1	3.4	3.4	53 ± 9.8
Tramadol	Low	2.4	4.3	5.2	107 ± 21
	Med	0.4	4.5	4.5	N/A
	High	4.9	1.8	2.3	183 ± 90
Detomidine	Low	3.9	7.6	7.7	88 ± 24
	Medium	5.9	7.7	9.1	N/A
	High	2.9	4.5	5.9	79 ± 12
Butorphanol	Low	−0.4	10.2	15.2	87 ± 12
	Medium	1.9	4.5	4.5	N/A
	High	−1.8	4.0	6.6	84 ± 10
Buprenorphine	Low	−9.3	4.5	8.0	87 ± 23
	Medium	−1.2	4.5	4.5	N/A
	High	−0.6	2.0	2.0	88 ± 7.7
Acepromazine	Low	87	28	53	97 ± 31
	Medium	8.3	8.2	11	N/A
	High	−0.7	11	11	117 ± 14
Flocoxib	Low	4.7	3.0	6.7	82 ± 11
	Medium	2.6	1.7	2.0	N/A
	High	2.8	4.0	4.0	83 ± 8.7
Meloxicam	Low	4.1	2.9	5.7	78 ± 12
	Medium	4.2	3.1	5.2	N/A
	High	−4.1	3.1	3.5	70 ± 12
Chlorpromazine	Low	−3.5	3.2	6.3	77 ± 42
	Medium	6.2	2.9	2.9	N/A
	High	0.9	2.9	3.2	90 ± 41
Ketoprofen	Low	6.1	5.6	7.0	78 ± 13
	Medium	−1.9	1.8	2.1	N/A
	High	5.3	5.5	5.5	86 ± 12
Flunixin	Low	5.8	5.8	7.3	89 ± 16
	Medium	0.4	2.2	2.8	N/A
	High	3.5	3.2	3.2	75 ± 7.0
Fluphenazine	Low	7.3	5.6	14.8	92 ± 125
	Medium	12.2	20	21	N/A
	High	18.4	18.7	19.3	81 ± 51
Phenylbutazone	Low	6.1	9.8	10.7	75 ± 14
	Medium	1.2	4.4	6.1	N/A
	High	2.3	4.6	6.2	60 ± 6.7

observed localization pattern with a peak concentration of 3600 pg/mg in the 16–18-cm segment is explained with known phenylbutazone administrations 10 months before sampling. Finally, meloxicam was detected in the tail hair of horse P02 at levels close to the LLOQ (data not shown). Ketoprofen had been administered in two cases but without leaving any detectable residues in hair.

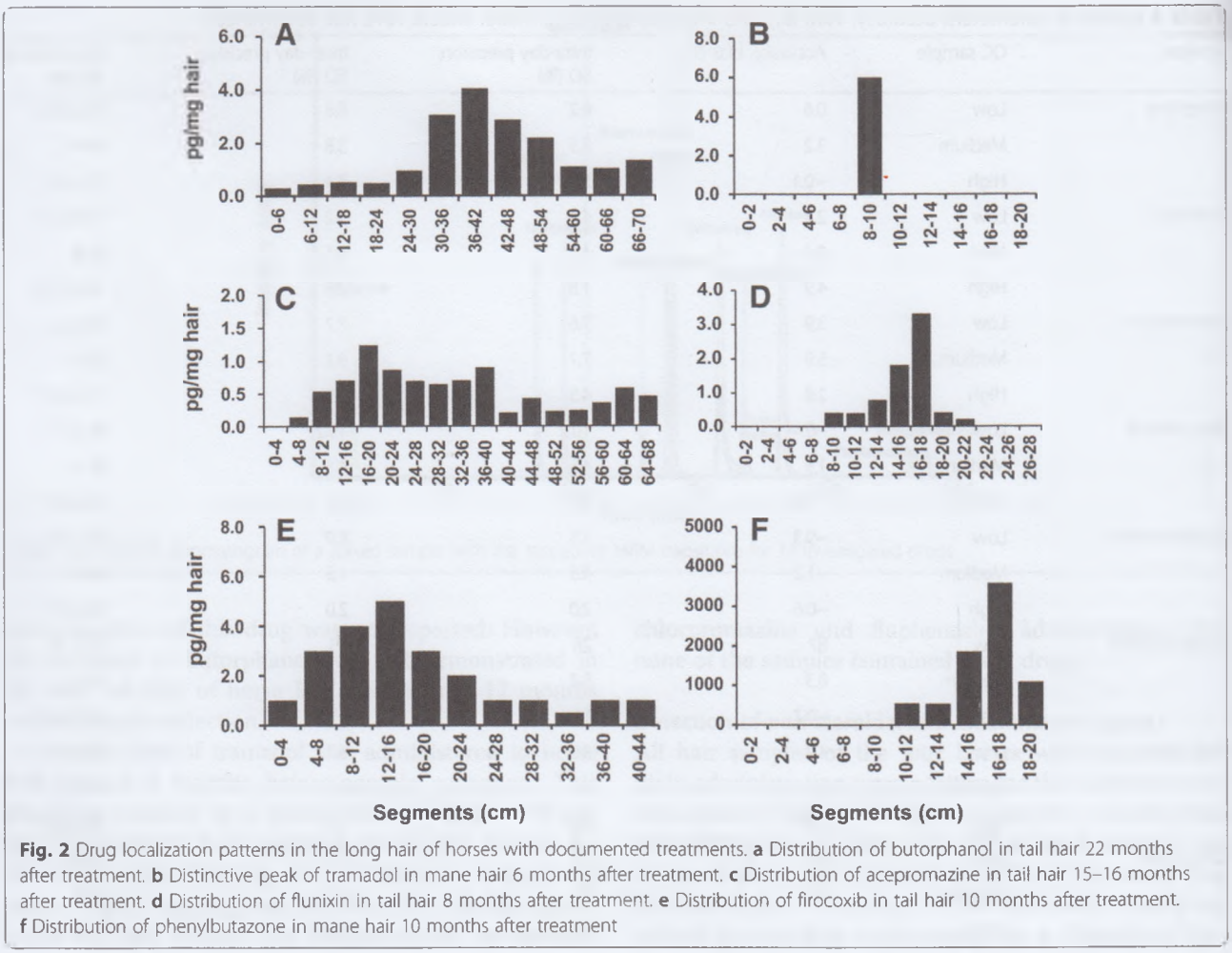

Fig. 2 Drug localization patterns in the long hair of horses with documented treatments. **a** Distribution of butorphanol in tail hair 22 months after treatment. **b** Distinctive peak of tramadol in mane hair 6 months after treatment. **c** Distribution of acepromazine in tail hair 15–16 months after treatment. **d** Distribution of flunixin in tail hair 8 months after treatment. **e** Distribution of firocoxib in tail hair 10 months after treatment. **f** Distribution of phenylbutazone in mane hair 10 months after treatment

Influence of pigmentation

The analyzed hair samples differed in color. The mane and tail hair of horse P01 as well as the mane hair of horse P09 were light and white, respectively, due to their low melanin content. In view of the melanin-dependent incorporation of basic drugs into hair (see Discussion), it is not surprising to find that the light hair of horse P01 was negative for all tested analytes although acepromazine and phenylbutazone administrations had been documented. Notably, the white mane hair of horse P09 (Fig. 3a) displayed only moderately lower concentrations of flunixin than the corresponding black tail hair of the same horse (Fig. 3b). However, all examined hair segments contained flunixin despite the fact that this drug was applied only 1 week before sample collection. Flunixin incorporated via the hair follicle could not yet have grown out as the follicle is located a few millimeters below the epidermis. Thus, the most likely explanation for the presence of flunixin in mane and tail hair of this horse is an incorporation via sweat and sebum [20].

Correlation between time of treatment and hair segment

Frequently, the analysis of mane or tail hair segments revealed a clear peak of drug incorporation (see for example Fig. 2b). In case of a broader distribution, as for example in Fig. 2c, the median distance from the skin was taken as the measure for drug localization. Subsequently, the assignments of drug localization in hair were compared to the reported times of treatment (Fig. 4). This relationship demonstrated that the time of drug administration can be calculated by assuming an average hair growth rate of 1.84 cm per month.

Discussion

The incorporation of a systemically administered drug into hair is dependent on the melanin content and the physicochemical properties of each substance [8, 21–23]. Basic drugs can reach 10-fold higher concentrations in pigmented than in non-pigmented hair [8, 21–23]. In contrast, no differences in hair incorporation were observed for acidic compounds and, as a consequence, hair concentrations of ketoprofen ($pk_a = 4.45$), meloxicam

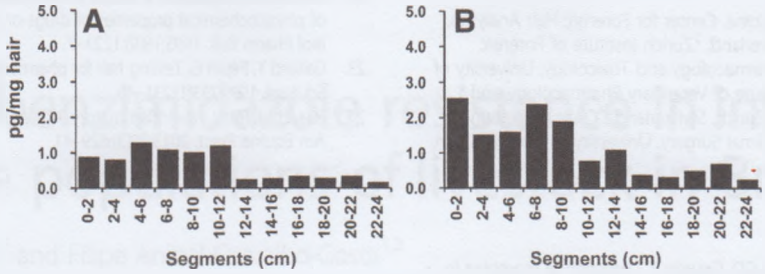

Fig. 3 Pattern of flunixin in hair of the same horse treated 1 week before sample collection. **a** Flunixin in white mane hair. **b** Flunixin in dark tail hair. These findings reflect sweat- and sebum-mediated flunixin incorporation

(pk_a = 4.05) or phenylbutazone (pk_a = 4.50) are low independently of hair color [8].

Several further considerations are necessary for the correct interpretation of hair findings. First, shifting of single hair against each other during sampling should be avoided to obtain the highest time resolution. Second, external contamination has to be taken into account and decontamination of hair samples is crucial. Third, one must consider that a substance may not only be found in the hair segment corresponding to the time of intake but also in adjacent segments. This may especially be the case when hair is cut into short segments of 1 to 2 cm length [8]. Fourth, the detection of substance in hair segments grown before drug administration can be explained by deposition mediated by sweat or sebum. Finally, it takes time for a hair sample to become negative after drug administration has been ceased due to the hair follicle proportion in the telogenic phase. In addition, hair follicle activity is compromised by disease or stressful events like anesthesia [24]. Decomposition of incorporated drug due to exposure to UV light or hair damage with drug leakage may account for negative findings [2].

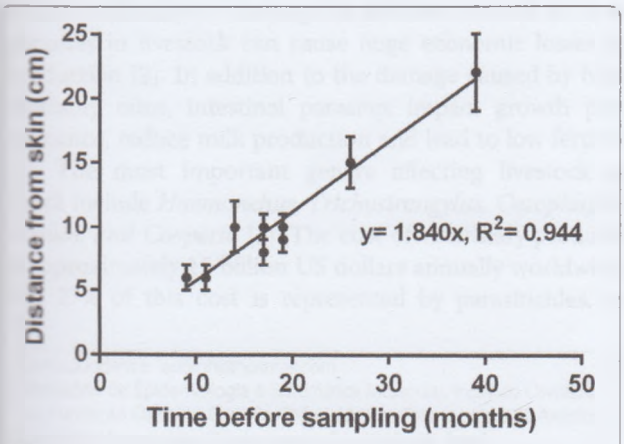

Fig. 4 Drug localization in horse hair as a function of the time of documented drug administrations. This graph shows the median distance from skin and range of segments with clearly increased drug incorporation. The slop of the resulting linear relationship is consistent with an average growth rate of 1.84 cm per month

Conclusion

In conclusion, the present fully validated LC-MS/MS method offers a valuable tool for the retrospective tracking of drugs as their detection in a hair segment proves previous medical treatments dating back up to 22 months before sampling. It was demonstrated that, particularly but not exclusively, drugs with neutral or basic properties can be monitored in dark long hair of horses. Further, our study demonstrates that the time of drug administration can be estimated based on an average growth rate of 1.84 cm per month. Further investigations should include the analysis of metabolites. Moreover, it should be determined to what extent light hair displaying a low melanin content could also be used for drug monitoring.

Abbreviations

LC-MS/MS, liquid chromatography tandem mass spectrometry; SoHT, society of hair testing; QC, quality control; MRM, multiple reaction monitoring; LOD, limit of detection; LLOQ, lower limit of quantification

Acknowledgments

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Authors' contributions

Sample collection, study design and documentation of drug administrations: MMM, JK, AF. Method development and sample analysis: MMM, BS. Interpretation, preparation of the manuscript: MMM, MRB, TK, JK. Initiation of the study and final manuscript version: HN. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Status of benzimidazole resistance in intestinal nematode populations of livestock in Brazil

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Abstract

Background: Benzimidazoles (BZ) are a class of drugs widely used in veterinary and human medicine, creating a great selection pressure and the emergence of BZ resistance. We conducted a systematic review to assess the status of resistance and/or effectiveness reduction of BZ drugs in animal nematodes in Brazil, and make information accessible to the scientific community, as many studies are published in Portuguese. PubMed, SciELO Brasil, LILACS/Bireme, GNTD database, and Google Scholar were searched with no language restrictions.

Results: A total of 40 studies met our eligibility criteria (from the year 1989 forward). Sheep was the host most frequently analysed, and albendazole was the most frequently drug studied. The majority of studies (75.7%) showed that BZ drugs are insufficiently active (FECRT <80%) against nematode parasites of livestock. The mean FECRT for fenbendazole, thiabendazole, albendazole, mebendazole, oxfendazole, and ricobendazole were 71.8%, 71.8%, 58.6%, 53.9%, 46.9%, and 41.5%, respectively. It was observed through linear regression that FECRT is significantly reduced over time between 2007 and 2014 ($R = -0.653$ $p = 0.021$) for the treatment of cattle with BZ, suggesting progressive loss of effectiveness and increased resistance for these hosts.

Conclusions: The scenario of BZ resistance in nematode populations in Brazil is not favourable. Given the high cost of drug discovery and development, it is urgent to implement control measures and to monitor the effectiveness/resistance to nematodes in livestock in Brazil.

Keywords: Benzimidazole, Anthelmintic resistance, Nematodes, Livestock, Brazil

Background

Livestock production is undermined by intestinal parasitic diseases [1]. The high prevalence of parasitic infections and the difficulty of carrying out effective control of these parasites in livestock can cause huge economic losses in production [2]. In addition to the damage caused by high mortality rates, intestinal parasites impact growth performance, reduce milk production and lead to low fertility [3]. The most important genera affecting livestock in Brazil include *Haemonchus, Trichostrongylus, Oesophagostomum, and Cooperia* [3]. The cost of veterinary products is approximately 15 billion US dollars annually worldwide, and 27% of this cost is represented by parasiticides. In Brazil, parasiticide purchases constitute 42% of the total volume of veterinary sales, representing 700 million US dollars annually [3]. Brazilian cattle herds reached 211,764 million animals in 2013, comprising the world's largest commercial herd. Brazilian sheep and goat herds comprised 17,291 million and eight million animals, respectively [4].

Benzimidazoles (BZ) are a class of drugs with activities against fungi, protozoa, and helminths [5, 6] and are widely used in veterinary and human medicine. The introduction of thiabendazole (THI) in the 1960s - the prototype of the first generation of benzimidazoles - provided a breakthrough in the treatment of diseases, allowing for the development of several other members of this class [5]. The primary mode of action of these drugs involves their interaction with the cytoskeletal protein β-tubulin, which together with α-tubulin constitutes the main component of microtubules [7].

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The BZ drugs have many benefits, including the following: i) selectivity and relatively low mammalian toxicity; ii) broad spectrum of activity; iii) high efficacy; iv) ease of administration; and v) low cost [5, 7–9]. For this reason, BZ drugs are widely used in livestock and are currently being employed in human MDA strategies. The success of anthelmintic treatment in the management and control of parasitic infections in livestock in the years following the development of BZ led to frequent and indiscriminate use of these drugs, thereby creating a great selection pressure in multiple species of nematodes [10]. This has the potential to select for parasite genotypes that are resistant to anthelmintics [11]. Drug resistance in any organism is defined by a change in the drug's pharmacokinetics and pharmacodynamics (absorption, distribution, metabolism, excretion, and site of action) [5] that allows some individuals in a population to tolerate doses of a given compound that would not normally be tolerated.

In this systematic review, we assess the status of resistance and/or effectiveness reduction of benzimidazole drugs in livestock nematodes in Brazil to review the history of BZ resistance in the country, generate data to enable monitoring and verification of the spread of BZ resistance, and make information accessible to the scientific community, as many studies are published in Portuguese.

Methods

Data sources and inclusion/exclusion criteria

Surveys assessing BZ resistance pertaining to animal intestinal nematodes in Brazil were extracted from five electronic databases: PubMed/NCBI (US National Library of Medicine National Institutes of Health/National Center for Biotechnology Information Search database), SciELO Brasil (Scientific Electronic Library Online), LILACS-Bireme (*Biblioteca Virtual em Saúde* – BIREME/PAHO/WHO), GNTD database (Global Neglected Tropical Diseases database), and Google Scholar. The search was performed on November 21st, 22nd, and 28th, 2015, using the terms: "resistance", AND "benzimidazole", OR "albendazole", OR "mebendazole", AND "Brazil". No language restrictions were made. Duplicate papers were removed. The PRISMA guideline/checklist was used to construct the systematic review [12].

Studies were eligible for inclusion if they met the following criteria: i) evaluated the BZ resistance/efficacy in nematode parasites in livestock hosts; ii) studied natural infections; iii) showed BZ resistance/efficacy of at least one BZ anthelmintic; iv) used at least one technique to detect BZ resistance/effectiveness; and v) were published in scientific journals with an International Standard Serial Number (ISSN). Congress abstracts, theses, and dissertations were not included. The exclusion criteria were as follows: i) articles that explored the BZ resistance only associated with other drug classes (e.g., BZ + macrocyclic lactones and other associations); ii) studies evaluating BZ resistance on fungi or other microorganisms; and iii) works demonstrating only experimental infections or in vitro tests.

"Grey literature" was accessed to enrich the text but was not included in the systematic review.

Data extraction, analysis, and quality assessment

Once selected, the following data were extracted from each paper and entered into a Microsoft Office Excel database: author names, journal, publication year, language, state and city in which the study was performed, host types, number of hosts, BZ drugs, parasitological techniques, counts of eggs per gram (epg) of faeces, parasites genus/species found, control group, if animal was dewormed and for how long, BZ resistance-related single nucleotide polymorphisms (SNPs) found, efficacy, cure rate, and reinfection rate. The studies were categorized into five quality levels (1 to 5; data not shown) based on the detail of the herds, number of animals evaluated, drugs evaluated, number and quality of the parasitological techniques used, and FECRT calculation.

Faecal egg count reduction test analysis

An assessment of treatment efficacy was performed by analysing the **F**aecal **E**gg **C**ount **R**eduction **T**est (FECRT syn. Egg reduction rate/ERR) results. When the studies did not present FECRT results, the values were calculated based on the eggs per gram of faeces before and after treatment, according to [13]. For the interpretation of the FECRT results in livestock, the following criteria were used: FECRT > 98%, highly effective; FECRT 90–98%, effective; FECRT 80–89%, moderately effective; and FECRT < 80%, insufficiently active [14].

The SPSS® Statistic Software v.20 (IBM Corp., Armonk, USA) was used to simple linear regression analysis, with a statistical significance of 5% ($p = 0.05$). We employed the general software Diva-GIS v.7.5.0.0 for map construction (downloaded free from the website: http://www.diva-gis.org).

Results

The search resulted in the gathering of 9176 files (articles or other texts). After applying the inclusion and exclusion criteria, 40 scientific articles were selected (Table 1). Most of the studies were published in Portuguese (23/40, 57.5%), and 17 (42.5%) studies were published in English. This review includes articles conducted from the year 1989 forward.

Data from 13 Brazilian states were analysed in the studies (Fig. 1). The largest number of studies was performed in Northeast (14/40, 32.6%), followed by Southeast (32.5%), South (30.0%), and Centre West (2.5%). The states most frequently analysed were the following: São

Table 1 List of studies assessing benzimidazole resistance in livestock hosts in Brazil, from 1989 to 2015

Author[a] year [reference number] Journal	State	BZ drug	Host	Diagnostic Approach			Nematode genus
				Parasitological technique		Molecular technique (SNP detection)	
				McMaster epg	Culture		
Alves et al. 2007 [43] Acta Veterinaria Brasilica	AL	ALB	Goat	Y	Y	N	*Haemonchus, Strongyloides*
Amarante et al. 1992 [44] Brazilian Journal of Veterinary	SP	OXF	Sheep	Y	Y	N	*Haemonchus, Trichonstrongylus*
Araujo et al. 2008 [45] Acta Veterinaria Brasilica	RN	RIC	Equine	Y	N	N	NA
Borges et al. 2010 [46] Ciencia Animal Brasileira	PR	OXF	Equine	Y	Y	N	Cyathostominae
Borges et al. 2015 [15] Pesquisa Veterinária Brasileira	BA	ALB	Goat	NI	Y	N	*Haemonchus, Trichonstrongylus*
Brasil et al. 2012 [47] International Journal for Parasitology	MG SP SC	ALB	Cattle Goat Sheep	Y	Y	Y	*Haemonchus*
Bruhn et al. 2012 [48] Acta Tecnológica	MG	ALB	Cattle	Y	N	N	NA
Castro et al. 2010 [16] Veterinary Parasitology	RS	ALB	Sheep	Y	Y	N	*Haemonchus, Trichonstrongylus, Ostertagia*
Coelho et al. 2010 [17] Ciência Animal Brasileira	RN	ALB	Goat	Y	Y	N	*Haemonchus, Trichonstrongylus*
Cunha Filho et al. 1998 [49] Semina Ciências Agrárias	PR	ALB	Sheep	Y	Y	N	*Haemonchus, Strongyloides, Trichonstrongylus, Ostertagia, Oesophagostomum, Cooperia, Bunostomum*
da Cruz et al. 2010 [18] Veterinary Parasitology	RJ	ALB	Sheep	Y	N	N	NA
dos Reves et al. 2014 [30] Veterinary Parasitology	SP	ALB	Cattle	Y	Y	N	*Haemonchus, Trichonstrongylus, Oesophagostomum, Cooperia*
de Souza et al. 2012 [50] Veterinary Parasitology	MG	ALB	Ostrich	Y	Y	N	*Libyostrongylus*
dos Santos et al. 2014 [51] Veterinary Parasitology	CE	OXF	Sheep	Y	Y	Y	*Haemonchus, Trichonstrongylus, Oesophagostomum*
Duarte et al. 2012 [52] Pesquisa Veterinária Brasileira	MG	ALB	Sheep	Y	Y	N	*Haemonchus, Strongyloides, Trichonstrongylus, Oesophagostomum, Cooperia*
Echevarria et al. 1996 [53] Veterinary Parasitology	RS	ALB	Sheep	NI	Y	N	*Haemonchus, Trichonstrongylus, Ostertagia*
Farias et al. 1997 [54] Veterinary Parasitology	SP	ALB MEB OXF	Sheep	Y	Y	N	*Haemonchus, Trichonstrongylus, Ostertagia*
Hammerschmidt et al. 2012 [20] Brazilian Journal of Veterinary Research and Animal Science	SC	ALB	Goat	Y	Y	N	*Haemonchus, Trichonstrongylus, Oesophagostomum*

Table 1 List of studies assessing benzimidazole resistance in livestock hosts in Brazil, from 1989 to 2015 *(Continued)*

Author [a] year [reference number] Journal	State	BZ drug	Host	Diagnostic Approach			Nematode genus
				Parasitological technique		Molecular technique (SNP detection)	
				McMaster epg	Culture		
Klauck et al. 2014 [55] Annals of the Brazilian Academy of Sciences	SC	ALB	Sheep	Y	Y	N	*Haemonchus, Trichostrongylus, Cooperia, Teladorsagia*
Lima et al. 2010 [56] Ciência Animal Brasileira	PE	ALB	Goat Sheep	Y	Y	N	*Haemonchus, Trichonstrongylus, Oesophagostomum*
Lima et al. 2010 [57] Pesquisa Veterinária Brasileira	PE	ALB	Goat	Y	Y	N	*Haemonchus, Strongyloides, Trichonstrongylus, Oesophagostomum*
Melo et al. 1998 [58] Ciência Animal	CE	OXF	Sheep	Y	Y	N	*Haemonchus, Trichonstrongylus, Cooperia, Trichuris*
Melo et al. 2003 [59] Ciência Rural	CE	OXF	Goat Sheep	Y	Y	N	*Haemonchus, Trichonstrongylus, Oesophagostomum*
Niciura et al. 2012 [60] Veterinary Parasitology	SP	ALB	Sheep	N	N	Y	*Haemonchus*
Nunes et al. 2013 [61] Revista Brasileira de Parasitologia Veterinaria	MG SP	ALB	Cattle Goat Sheep	Y	N	Y	*Haemonchus*
Pereira et al. 2008 [62] Acta Veterinaria Brasilica	RN	ALB	Goat Sheep	NI	Y	N	*Haemonchus, Strongyloides, Trichonstrongylus, Oesophagostomum*
Ramos et al. 2002 [63] Ciência Rural	SC	ALB	Sheep	Y	Y	N	*Haemonchus, Trichonstrongylus, Ostertagia*
Rodrigues et al. 2007 [64] Pesquisa Veterinaria Brasileira	PB	ALB	Goat	Y	Y	N	*Haemonchus*
Santos et al. 2014 [65] Revista Portuguesa de Ciências Veterinarias	RS	OXF	Cattle	Y	Y	N	*Haemonchus, Trichonstrongylus, Ostertagia, Cooperia, Bunostomum*
Sczesny-Moraoes et al. 2010 [66] Pesquisa Veterinária Brasileira	MS	ALB	Sheep	Y	Y	N	*Haemonchus, Strongyloides, Trichonstrongylus, Cooperia*
Soutelo et al. 2007 [67] Veterinary Parasitology	SP	ALB	Cattle	NI	Y	N	*Haemonchus, Trichonstrongylus, Oesophagostomum, Cooperia*
Soutelo et al. 2010 [68] Revista Brasileira de Parasitologia Veterinária	SP	ALB	Cattle	Y	Y	N	*Haemonchus, Oesophagostomum, Cooperia*
Souza et al. 2008 [69] Ciência Rural	SC	ALB	Cattle	Y	Y	N	*Cooperia*
Souza et al. 2013 [70] Agropecuária Científica do Semiárido	PB	ALB	Goat	Y	N	N	NA
Thomas-Soccol et al. 1996 [21] Veterinary Record	PR	ALB	Sheep	Y	N	N	NA
Thomas-Soccol et al. 2004 [22] Brazilian Archives of Biology and Technology	PR	OXF	Sheep	NI	Y	N	*Haemonchus, Trichonstrongylus, Oesophagostomum, Ostertagia, Cooperia*

Table 1 List of studies assessing benzimidazole resistance in livestock hosts in Brazil, from 1989 to 2015 *(Continued)*

Author [a] year [reference number] Journal	State	BZ drug	Host	Diagnostic Approach			Nematode genus
				Parasitological technique		Molecular technique (SNP detection)	
				McMaster epg	Culture		
Veríssimo et al. 2012 [71] Veterinary Parasitology	SP	ALB	Sheep	NI	Y	N	*Haemonchus, Strongyloides, Trichonstrongylus, Oesophagostomum, Cooperia*
Vieira and Cavalcante 1999 [72] Pesquisa Veterinária Brasileira	CE	OXF	Goat	Y	Y	N	*Haemonchus, Oesophagostomum*
Vieira et al. 1989 [73] Pesquisa Agropecuária Brasileira	CE	ALB FEN OXF THI	Goat	NI	Y	N	*Haemonchus, Trichonstrongylus, Oesophagostomum*
Vieira et al. 1989 [74] Boletim de Pesquisa Embrapa	CE	ALB FEN OXF THI	Goat Sheep	NI	Y	N	*Haemonchus, Strongyloides*

Total = 40

[a] listed alphabetically. *ALB* albendazole, *FEN* fenbendazole, *MEB* mebendazole, *OXF* oxfendazole, *RIC* ricobendazole, *THI* thiabendazole. *Epg* eggs per gram of faeces, *AL* Alagoas, *BA* Bahia, *CE* Ceará, *MG* Minas Gerais, *MS* Mato Grosso do Sul, *PB* Paraíba, *PE* Pernambuco, *PR* Paraná, *RJ* Rio de Janeiro, *RN* Rio Grande do Norte, *RS* Rio Grande do Sul, *SC* Santa Catarina, *SP* São Paulo. *Y* Yes, *N* No, *NI* Not informed, *NA* Not applied

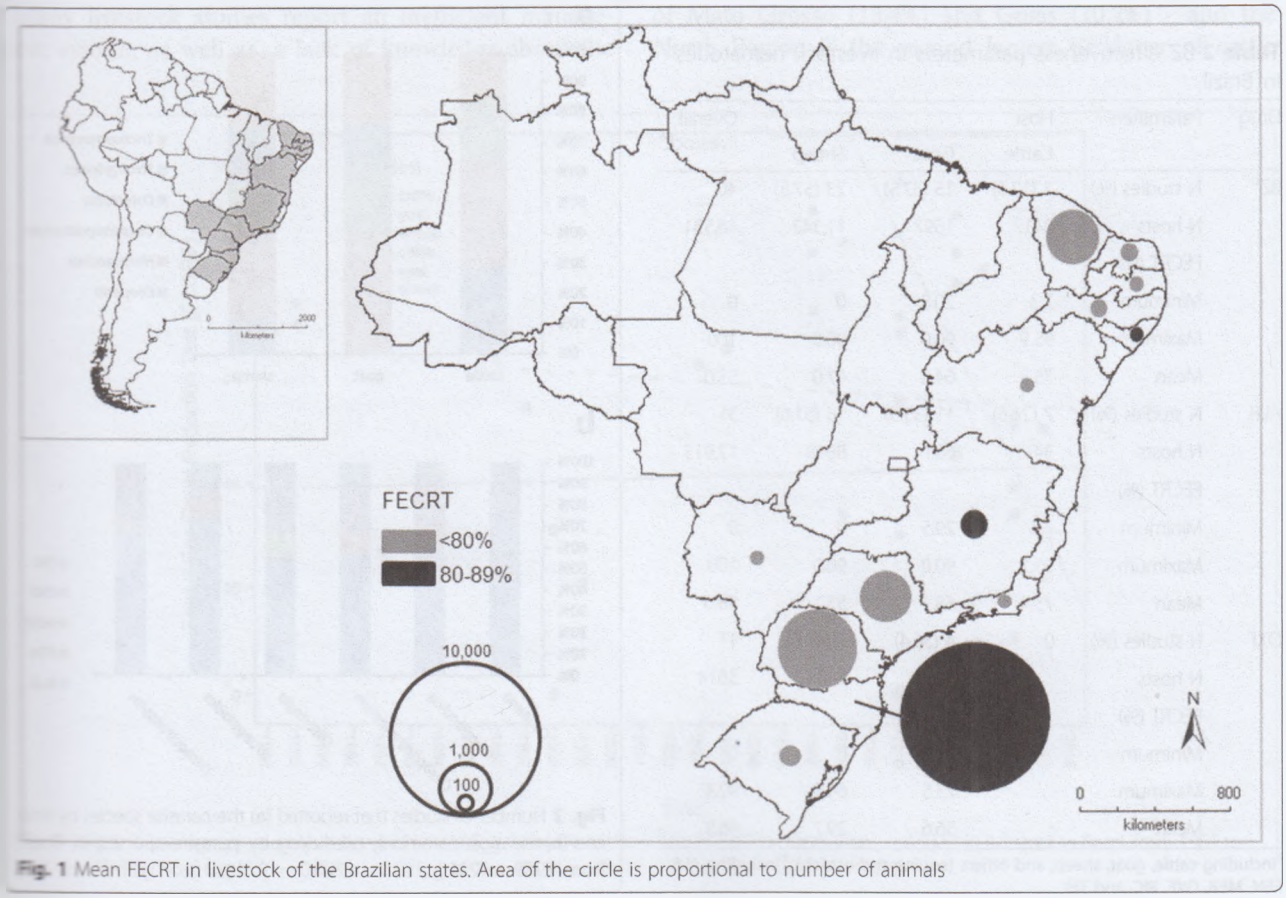


Fig. 1 Mean FECRT in livestock of the Brazilian states. Area of the circle is proportional to number of animals

Paulo (9/40, 22.5%), Ceará (15.0%), and Minas Gerais and Santa Catarina (12.5%) (Table 1).

In livestock, sheep were the host most frequently analysed (23/40, 57.5%) (Table 2), followed by goats (37.5%), cattle (17.5%), and others (equines and ostrich, 7.5% each). The BZ drugs tested in livestock were ALB (31/40, 72.1%), oxfendazole (OXF) (27.5%), fenbendazole (FEN) (4.7%), THI (4.7%), and MEB (2.3%), and ricobendazole (RIC) (2.3%).

The majority of studies (75.7%) showed that BZ drugs are insufficiently active (FECRT <80%) against nematode parasites of livestock (Fig. 1). The mean FECRT for BZ drugs was 55.0% (Table 2); the mean FECRTs for FEN and THI was of 71.8%, the mean for ALB was 58.6%, the mean for MEB was 53.9%, the mean for OXF was 46.9%, and the mean for RIC was 41.5%. Five studies showed FECRTs lower than 1%. It is noteworthy that the most studied animal – sheep – showed the lowest mean FECRT for BZ drugs (mean FECRT = 47.0%) (Table 2). Only one study (2.5%) demonstrated that ALB is highly effective (FECRT > 98%) in ostrich against the nematode genus *Libyostrongylus*.

Among the techniques used to perform the eggs counts, the Gordon and Whitlock technique associated with the McMaster chamber was the most frequently used (75.0% of studies). The egg hatch test, as well as the FLOTAC technique, was used in only one study. Coproculture was performed in 33/40 (82.5%) of the studies to identify the nematode genus through morphological analyses of the larvae. Through coproculture, the following parasite genera were identified (Table 1): *Haemonchus* (32.2%), *Trichostrongylus* (21.7%), *Oesophagostomum* (16.1%), *Cooperia* (9.6%), *Strongyloides* (8.8%), *Ostertagia* (5.6%), and others *(Strongylus, Bunostomum, Teladorsagia, Trichuris, Libyostrongylus*, 5.6%). Sheep exhibited a greater diversity of parasites compared to other animal hosts (Fig. 2a), with the following genera identified: *Cooperia, Haemonchus, Oesophagostomum, Ostertagia, Strongyloides,* and *Trichostrongylus*. In Fig. 2b, it noted the number of studies that identified the parasite species and the studied drug. However, it was not possible to establish a relationship between a specific parasite genera and BZ effectiveness. Four studies (9.3%) used molecular techniques to evaluate potential BZ resistance. The characteristic mutation at codon F200Y of the beta-tubulin gene was the most frequently observed mutation (100%) in *Haemonchus* parasites, yet the F167Y mutation in the same gene was found in only two studies (50%).

Table 2 BZ effectiveness parameters in livestock nematodes in Brazil

Drug	Parameters	Host			Overall livestock[a]
		Cattle	Goat	Sheep	
BZ[b]	N studies (%)	7 (17.5)	15 (37.5)	23 (57.5)	40
	N hosts	3417	1697	11,342	16,531
	FECRT (%)				
	Minimum	7.3	20.8	0	0
	Maximum	95.9	90.0	90.0	100
	Mean	75.3	64.8	47.0	55.0
ALB	N studies (%)	7 (22.6)	11 (35.5)	16 (51.6)	31
	N hosts	3417	604	8878	12,915
	FECRT (%)				
	Minimum	7.3	29.5	0	0
	Maximum	95.7	90.0	90.0	100
	Mean	75.4	68.1	55.1	58.6
OXF	N studies (%)	0	4 (36.4)	7 (63.6)	11
	N hosts		1118	2464	3614
	FECRT (%)				
	Minimum		20.8	0	0
	Maximum		73.5	64.9	92.4
	Mean		56.6	29.7	46.9

[a]including cattle, goat, sheep, and others (equine and ostrich). [b]including ALB, FEN, MEB, OXF, RIC, and THI

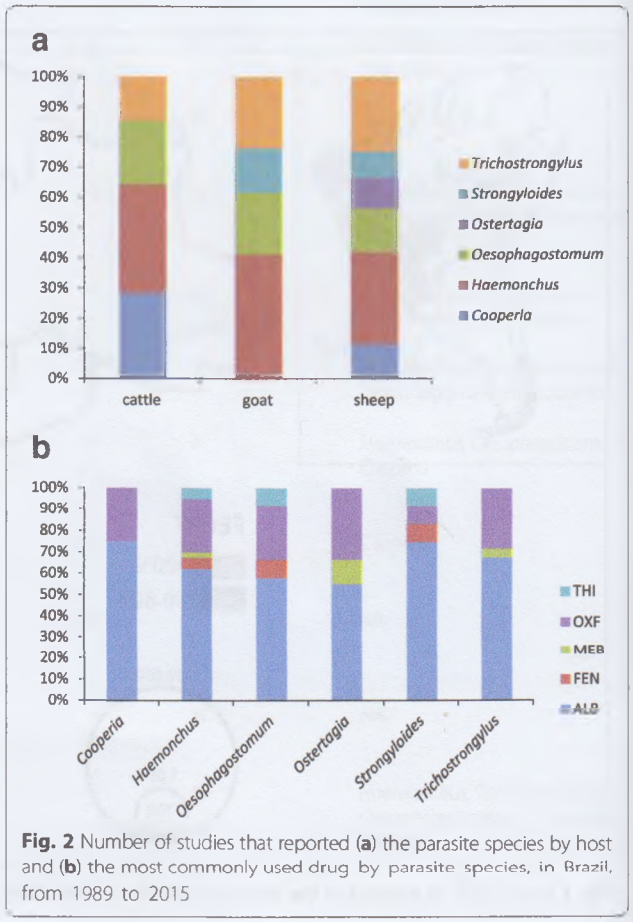


Fig. 2 Number of studies that reported (**a**) the parasite species by host and (**b**) the most commonly used drug by parasite species, in Brazil, from 1989 to 2015

...was observed through linear regression that FECRT ...significantly reduced over time between 2007 and ...4 ($R = -0.653$ $p = 0.021$) for the treatment of cattle ...BZ, suggesting progressive loss of effectiveness and ...creased resistance for these hosts (Fig. 3).

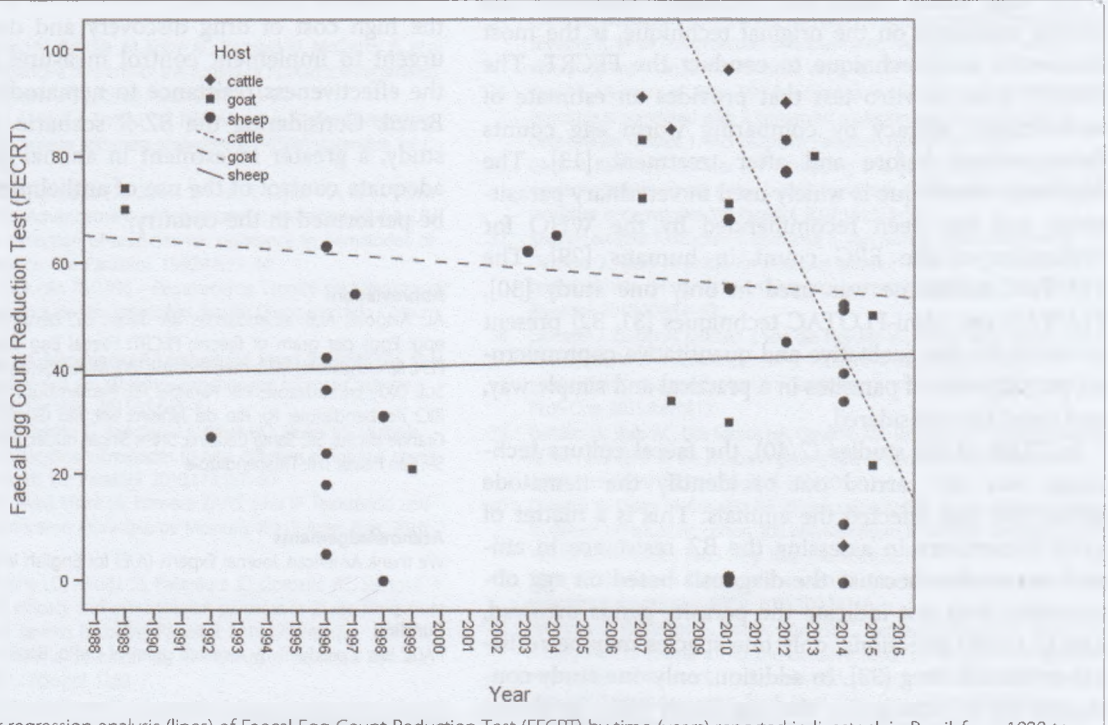
Discussion

This study presents a systematic review on a subject still ...er-explored in Brazil: BZ resistance in nematode ...sites.

...e observed that BZ resistance was widely disseminated in animal hosts in Brazil and demonstrated that BZ had lower effectiveness in sheep. BZ resistance in livestock has been widely distributed throughout the world since the development of the drugs in the early 1960s [7]. The extensive use of BZ led to an immense infection pressure on parasite populations, particularly in the gastrointestinal parasites of ruminants, most notably sheep [7]. Grazing animals defecate where they feed, and even after treatment, reinfection is common, leading to an overuse of BZs. In this context, anthelmintic drugs are often used as a single tool for nematode control, excessively and indiscriminately. Many farms provide various annual doses of BZ drugs to animals, allowing for a considerable selective pressure on parasitic nematodes, and the spread of resistance.

Many livestock studies report an inefficient management system, as well as, a lack of knowledge about the correct use and dosage of drugs and not respecting the interval time between dosage administrations [15–18]. This has a great impact on treatment efficacy. In Brazil, the SICOPA (*Sistema Integrado de Controle Parasitário*) [19] consists of a set of strategies for the treatment of the flock to preserve the drug susceptibility characteristics and considers the epidemiological characteristics of the country [20]. However, the monitoring of drug efficacy is rarely used or even non-existent on some farms in Brazil [19]. Therefore, some measures must be implemented in farms in order to reduce the selective pressure and the spread of resistance to anthelmintics: i) establish the parasitological diagnosis; ii) determine the FECRT routinely, as well as the susceptibility of the host population (naïve, preparturition, post parturition); iii) weigh the animals to avoid underdosage; iv) anthelmintic drug rotation (annually); and v) anthelmintic treatments not administered at intervals shorter than 28 days [19–23].

Brazil is a major producer of animals and meat for exportation to the world market, approximately 230 million animals are produced annually [4]. Nonetheless, only 13 of the 27 Brazilian states were analysed for BZ resistance. Cattle production is well distributed in the country, especially in the states of the Midwest (33.6% of total production in the country) - specifically the states of Mato Grosso (13.4%) and Goiás (10.2%) - and the North Region is the second largest producer of cattle



...3 Simple linear regression analysis (lines) of Faecal Egg Count Reduction Test (FECRT) by time (years) reported in livestock in Brazil, from 1989 to ...fied by host. Cattle $R = -0.653$ $p = 0.021$; goat $R = -0.154$ $p = 0.633$; and sheep $R = 0.029$ $p = 0.820$)

(21.2%) [4]. However, no work has been published reporting the effectiveness of BZ in these regions. Only one study was conducted in Mato Grosso do Sul (Midwest Region) and analysed sheep nematodes. The states of the Northeast and South Regions are the largest producers of sheep (56.5% and 30% of total production of the country, respectively), and the states of the Northeast are the main producers of goats, with 91.4% of the total production [4]. In these regions there was research available on BZ resistance of STHs in herds, both in sheep and goats. Nevertheless, the data shows us that there is a gap in knowledge - both in diagnosis and research - about the reality of resistance in livestock in the country.

The origin of BZ resistance in livestock has been speculated about. The animal migration and gene flow among nematodes [24], as well as spontaneous mutations [25] and the presence of rare alleles in the population [26], could be responsible for the spread of resistance among animal nematodes. Currently, there is concern about the possibility of the emergence of resistance to the drugs used in soil transmitted helminthes (STH) control; however, the large-scale mass drug administration strategy is generally the cornerstone of most STH control programmes [27, 28]. Until now, the degree of influence that resistance in livestock can have on the development and spread of resistance in human nematodes is unknown, particularly in nematodes with zoonotic potential, such as *Ascaris suum* and *Trichostrongylus*.

The egg count using the McMaster chamber, and several variations on the original technique, is the most frequently used technique to conduct the FECRT. The FECRT is an in vitro test that provides an estimate of anthelmintic efficacy by comparing worm egg counts from animals before and after treatment [13]. The McMaster technique is widely used in veterinary parasitology and has been recommended by the WHO for evaluation of the EPG count in humans [29]. The FLOTAC technique was used in only one study [30]. FLOTAC and Mini-FLOTAC techniques [31, 32] present potential for the qualitative and quantitative copromicroscopic diagnosis of parasites in a practical and simple way, and should be considered.

In 17.5% of the studies (7/40), the faecal culture technique was not carried out to identify the nematode genus that had infected the animals. This is a matter of great importance in assessing the BZ resistance in animal nematodes, because the diagnosis based on egg observation does not indicate the parasite genus involved, and in mixed infections, only one species may be resistant to the BZ drug [33]. In addition, only one study conducted an in vitro test – the egg hatch test - to detect the nematode species involved in BZ resistance. The egg hatch test can be used for detection of BZ resistance by assessing the drug's ability to inhibit embryonation of the parasite [13, 34].

The molecular signature of BZ resistance in nematodes is the presence of SNPs in the β-tubulin isotype 1 gene in nematodes, located at codons F167Y (TTC/Phe → TAC/Tyr), E198A (GAG/Glu → GCG/Ala) and F200Y (TTC/Phe → TAC/Tyr) [6, 35, 36]. Despite the fact that these genetic markers of BZ resistance are known, few studies (4/43, 9.3%) used molecular techniques for the evaluation of resistance of nematodes in Brazil. Of these studies, all *Haemonchus* nematodes demonstrated the mutation F200Y. Additionally, the codon F167Y was found in *Haemonchus* parasites in two studies. These findings in Brazil are in agreement with previous studies, which demonstrated that the F200Y mutation is the most frequently found mutation associated with BZ resistance in nematodes, and has been described in various nematode parasites: *Haemonchus* [36], *Ostertagia* [37], *Cooperia* [38], *Ancylostoma caninum* [39], human hookworms and *T. trichiura* [10, 40]. Moreover, *Haemonchus contortus* has been determined to be responsible for the rapid development of BZ resistance in nematodes of small ruminants, probably due to its high genetic diversity and consequent greater availability for new mutations [41, 42].

Conclusions

The scenario of BZ resistance in nematode populations of domestic animals in Brazil is not favourable. Given the high cost of drug discovery and development, it is urgent to implement control measures and to monitor the effectiveness/resistance to nematodes in livestock in Brazil. Considering the BZ-R scenario observed in this study, a greater investment in animal management and adequate control of the use of anthelmintic drugs should be performed in the country.

Abbreviations

AL: Alagoas; ALB: albendazole; BA: Bahia; BZ: benzimidazole; CE: Ceará; epg: Eggs per gram of faeces; FECRT: Faecal Egg Count Reduction Test; FEN: fenbendazole; MEB: mebendazol; MG: Minas Gerais; MS: Mato Grosso do Sul; OXF: oxfendazole; PB: Paraíba; PE: Pernambuco; PR: Paraná; RIC: ricobendazole; RJ: Rio de Janeiro; RN: Rio Grande do Norte; RS: Rio Grande do Sul; SC: Santa Catarina; SNPs: Single nucleotide polymorphisms; SP: São Paulo; THI: Thiabendazole

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Authors' contributions

LHJ and FACC conceived and designed the study. LHJ conducted the systematic literature search and data extraction. LHJ and FACC analyzed and interpreted the

data and wrote the manuscript. Both authors have read and approved the final version of this manuscript.

Competing interests
The authors declare that they have no competing interests.

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The method of attachment influences accelerometer-based activity data in dogs

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Abstract

Background: Accelerometer-based activity monitoring is a promising new tool in veterinary medicine used to objectively assess activity levels in dogs. To date, it is unknown how device orientation, attachment method, and attachment of a leash to the collar holding an accelerometer affect canine activity data. It was our goal to evaluate whether attachment methods of accelerometers affect activity counts. Eight healthy, client-owned dogs were fitted with two identical neck collars to which two identical activity monitors were attached using six different methods of attachment. These methods of attachment evaluated the use of a protective case, positioning of the activity monitor and the tightness of attachment of the accelerometer. Lastly, the effect of leash attachment to the collar was evaluated. For trials where the effect of leash attachment to the collar was not being studied, the leash was attached to a harness. Activity data obtained from separate monitors within a given experiment were compared using Pearson correlation coefficients and across all experiments using the Kruskal-Wallis Test.

Results: There was excellent correlation and low variability between activity monitors on separate collars when the leash was attached to a harness, regardless of their relative positions. There was good correlation when activity monitors were placed on the same collar regardless of orientation. There were poor correlations between activity monitors in three experiments: when the leash was fastened to the collar that held an activity monitor, when one activity monitor was housed in the protective casing, and when one activity monitor was loosely zip-tied to the collar rather than threaded on using the provided metal loop. Follow-up, pair-wise comparisons identified the correlation associated with these three methods of attachment to be statistically different from the level of correlation when monitors were placed on separate collars.

Conclusions: While accelerometer-based activity monitors are useful tools to objectively assess physical activity in dogs, care must be taken when choosing a method to attach the device. The attachment of the activity monitor to the collar should utilize a second, dedicated collar that is not used for leash attachment and the attachment method should remain consistent throughout a study period.

Background

Accelerometry has recently been introduced to veterinary medicine as a novel outcome measurement to objectively assess activity levels in dogs. This technology represents a valuable tool that has frequently been utilized in conjunction with other, previously validated outcome measures such as ground reaction forces and validated owner questionnaires [1–11]. Omnidirectional activity monitors measure spontaneous activity over an adjustable period of time ("epoch") and filter out constant sources of acceleration (i.e. gravity) [7].

Many studies have investigated the utility of accelerometer-based activity monitors for various applications, [1–19] particularly including the measurement of activity after various therapies for dogs with osteoarthritis [1, 3, 4, 16, 18, 19]. Interestingly, there are some instances where improvement of currently accepted outcome measures (e.g. owner questionnaires, gait analysis) was not accompanied by similar improvement in accelerometer-based activity levels [4, 15, 16, 18, 19]. The reason for this disconnect may be that these outcome measures evaluate different components of improvement associated with the

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successful treatment of osteoarthritis or that variables affecting activity data were not controlled for. Such reported variables include signalment, [2, 10, 15] body weight, [10, 15] body conformation, [2] and activity monitor positioning [8]. However, other factors such as method of accelerometer attachment to the collar have not been investigated. While many studies describe attaching the activity monitor to the collar of study participants [2–8, 10–12, 18], only few authors have provided detailed descriptions of how the activity monitor was specifically attached [5, 7, 8, 12]. Given the high sensitivity of these devices [12], it seems possible that factors such as leash attachment to the collar and tightness of monitor attachment to the collar may affect activity data. Furthermore, the use of a protective case (to extend waterproof capabilities and protect the costly activity monitors) has been previously described, [12] but the impact of this case on activity data is unknown. To the authors' knowledge, no studies have been conducted that investigate these factors as sources of activity data variability. Hence, it was our goal to identify how specific attachment methods might affect activity measurements. Specifically, our objective was to evaluate whether device orientation, use of a protective case, attachment of an activity monitor using zip ties, and connection of a leash to the collar holding the activity monitor would have a significant impact on total activity counts. We hypothesized that these factors would significantly affect activity data.

Methods

Animals

Client-owned dogs were recruited from faculty, staff, and students of the Colorado State University Veterinary Teaching Hospital. Each dog was deemed clinically healthy by owner history and thorough physical examination. The dogs were individually fitted with two identical neck collars[1] to which two identical activity monitors[2] were attached using six different methods of attachment as outlined below. The collars were adjusted to ensure a snug fit and the specific hole in the collar used to secure it was noted for each dog to ascertain that the amount of tension on the collar was consistent throughout the study period as previously reported [20]. Due to limitations in the ability to consistently attach activity monitors to excessively small or large collars, small and giant breed dogs were excluded from the study. The study protocol was approved by the Institutional Clinical Research Review Board (VCS#2015-029) and written owner consent was obtained.

Activity monitoring

A previously validated [1–11], omnidirectional accelerometer-based activity monitor was used in all dogs. Six methods of attachment were studied (Fig. 1) in seven different experiments. Eight dogs participated in the study (4 neutered males, 4 spayed females). The mean age ± SD was 3.8 ± 2.4 years (range: 1.2–8.7 years) and the mean weight ± SD was 21.3 ± 5.77 kg (range: 12.2–29.3 kg). Breeds included Border Collie ($n = 3$), mixed breed ($n = 2$), Labrador Retriever ($n = 1$), Golden Retriever ($n = 1$), and Husky ($n = 1$). Two separate accelerometers were first attached in identical fashion using the metal loops of the accelerometers to two separate, identical collars (Fig. 1a; Experiment 1). For this experiment, the leash attached to a harness[3]. Inter-collar rotation was subjectively monitored for during each data collection. If it was noted that inter-collar rotation occurred, that data set was discarded and data collection was repeated. The rostral/caudal position of these collars was then switched (Experiment 2). Next, the leash was attached to the rostral collar instead of the harness (Fig. 1b; Experiment 3). Accelerometers were then placed on the

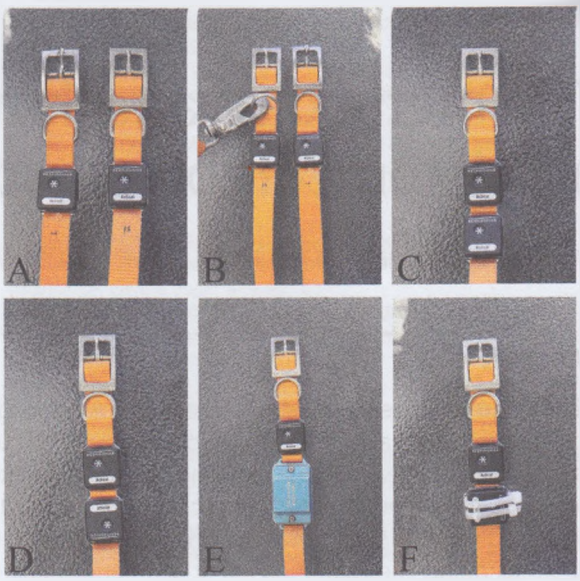

Fig. 1 Photographs illustrating the various methods of attachment utilized in this study. **a** The activity monitors were threaded onto separate collars using the provided metal loops on the monitors. This method of attachment was utilized in Experiments 1 & 2. **b** The activity monitors were threaded onto separate collars using the provided metal loops on the monitors and a leash was attached to the rostral collar. This method of attachment was utilized in Experiment 3. **c** The activity monitors were threaded onto the same collar in the same orientation using the provided metal loops on the monitors. This method of attachment was utilized in Experiment 4. **d** The activity monitors were threaded onto the same collar in opposite orientations using the provided metal loops on the monitors. This method of attachment was utilized in Experiment 5. **e** The activity monitors were placed on the same collar. One monitor was threaded on using the provided metal loop on the monitor. The other activity monitor was placed in the same orientation as the first, but inside a metal protective case on the same collar. This method of attachment was utilized in Experiment 6. **f** One activity monitor was attached using the provided metal loop on the monitor. The other monitor was rotated 90° and attached to the same collar using zip-ties. This method of attachment was utilized in Experiment 7

same collar in the same orientation and the second collar was removed (Fig. 1c; Experiment 4). The orientation of one accelerometer was then changed by rotating it 180° (Fig. 1d; Experiment 5). One accelerometer was then placed inside a protective case (provided by the manufacturer) with the same orientation as the other accelerometer on the same collar (Fig. 1e; Experiment 6). Finally, one accelerometer was rotated 90° and attached loosely with zip-ties to the same collar (Fig. 1f; Experiment 7). This trial necessitated the rotation of one accelerometer 90° as holes would have needed to be placed in the collar to facilitate the use of zip ties without rotation.

Three trials were collected for each dog in each experiment. A trial consisted of a 3-min outdoor leash walk across a concrete surface. The collars were oriented so that the activity monitors rested ventrally on the dog's neck. The epoch length was set to 1 s, resulting in approximately 180 data points for every trial. The same handler walked each dog in the same fashion (i.e. the same location and speed was kept subjectively consistent). For trials where the effect of leash attachment to the collar was not being studied, the leash was attached to a harness. To mark the beginning and end of each trial, the handler pressed the event marker button located on each activity monitor. Activity data was retrieved from the monitors using the provided communications interface[4] and software.

Data analysis

Post-collection processing of data sets included alignment of the starting points of each data set by visual analysis within a commercially available spreadsheet software.[5] Engagement of the event marker buttons on each accelerometer was identified within the spreadsheet program. If the data sets were not aligned, the timing was adjusted so that they were in alignment prior to statistical analysis. Statistical analysis was conducted using commercially available software.[6] The correlation of the average activity between accelerometers was evaluated by calculating the Pearson's correlation coefficient for each experiment. The correlation between accelerometers was compared across all experiments using a Kruskal-Wallis Test with post-hoc pairwise comparisons. Significance levels were adjusted using the Bonferroni correction.

Results

There was no statistically significant difference ($p > 0.05$) between data from individual trials in any given experiment, thus data from all trials for each dog were included in subsequent analysis; results are summarized in Table 1. There was excellent correlation and low variability between activity monitors on separate collars when the leash was attached to a harness, regardless of their relative positions (Experiments 1 & 2, CC > 0.9). There was also good correlation when activity monitors were placed on the same collar regardless of orientation (Experiments 4 & 5, CC > 0.75). However, confidence intervals for these experiments were wider than the experiments with activity monitors on separate collars when the leash was attached to a harness. Use of the protective case, leash attachment to the collar, and attachment with zip-ties resulted in the lowest correlations between collars (CC = 0.43, 0.62, 0.64 respectively). When correlation coefficients were compared across attachment methods, there was a statistically significant difference ($p < 0.0001$) in the level of correlation in these three experiments. Follow-up, pair-wise comparisons identified the correlation associated with these three methods of attachment to be statistically different ($p < 0.05$) from the level of correlation when activity monitors were placed on separate collars.

Discussion

Accelerometers have become a promising tool to objectively quantify both spontaneous and controlled physical activity in dogs. However, few studies have investigated the effect of the accelerometer positioning on the dog's collar. Our research identified the following methods of attachment to have a significant impact on the resultant activity data: (1) attaching a leash to the dog's collar that holds an activity monitor, (2) the use of a protective case and (3) attachment of the device loosely to the dog's collar using zip-ties. There was high correlation between activity monitors when they were on separate collars and the leash was attached to the harness, regardless of the relative collar position. This result was expected, as identical collars were used and there were no other confounding factors in this experiment. Additionally, there was strong correlation between activity monitors when they were on the same collar, regardless of orientation. Again, this was expected, as the activity monitors used in this study are omnidirectional and record activity data in all axes, irrespective of sensor orientation.

Non-ambulatory movements of the dog have previously been suggested as factors that could affect activity data [7, 14]. Similarly, the difference in activity data found in our study associated with leash attachment to the collar can easily be explained by additional or restricted movement of the collar caused by pull of the handler/leash and a different position of the accelerometer (dorsal compared to ventral) when tension is applied to the leash. Surprisingly, the majority of previous publications do not specify whether a separate collar was used to hold the activity monitor throughout the study period (Table 2). The findings of this study suggest that a second, dedicated collar should be utilized to attach an activity monitor. It is the authors' current practice and recommendation that the leash attachment ring (of the second, dedicated collar) should be removed to

Table 1 Description and correlation of attachment methods used in each experiment

Experiment	Device 1				Device 2				Mean Pearson's Correlation Coefficient	95% Confidence Interval
	Attachment	Collars	Leash	Collar Position	Attachment	Collars	Leash	Collar position		
1	Metal Loop	Separate	Harness	Rostral	Metal Loop	Separate	Harness	Caudal	0.918	0.883–0.953
2	Metal Loop	Separate	Harness	Rostral	Metal Loop	Separate	Harness	Rostral	0.932	0.905–0.958
3	Metal Loop	Separate	Rostral Collar	Caudal	Metal Loop	Separate	Rostral Collar	Rostral	0.615	0.467–0.764
4	Metal Loop	Same	Harness		Metal Loop	Same	Harness		0.786	0.639–0.933
5	Metal Loop	Same	Harness		Metal Loop –Flipped 180°	Same	Harness		0.76	0.603–0.917
6	Metal Loop	Same	Harness		Protective Case	Same	Harness		0.428	0.217–0.638
7	Metal Loop	Same	Harness		Zip-ties – Flipped 90°	Same	Harness		0.64	0.499–0.780

avoid any possibility of the dog's owner attaching a leash to the collar bearing an accelerometer.

The second method of attachment associated with poor correlation was when the metal protective casing provided by the manufacturer was used to house the activity monitor (Fig. 1e; Experiment 6). The accelerometer utilized in this study is currently priced at $450 and is rated for no greater than 1 meter of water submersion for 30 min. The protective case offers a simple way to extend the water-resistant capabilities of the device and protect it from incidental damage. However, our results indicate that the use of the protective case may affect activity data. These differences could be the result of the extra weight added to the activity monitor with the casing, providing it with more momentum to move on the neck. Alternatively, it is possible that the device shifts within the casing. It should also be noted that while using the protective case, the event marker buttons located on the activity monitors are inaccessible. These event marker buttons make it possible for owners to conveniently make note of significant events that occur while the dog is wearing the activity monitor, and so use of the protective casing may limit the usefulness of the monitor.

When two monitors were placed on the same collar in identical fashion (Fig. 1c & d; Experiments 4 & 5), lower than expected correlations were found between activity monitors. Furthermore, we did not find a significant difference between correlations of these experiments and when the device was placed in the protective case. This may indicate that the additional weight may play a role in accelerometer data acquisition. Based on these results, it seems that attaching two activity monitors to the same collar or adding any additional weight to the collar may affect activity data. However, further research is required to confirm these hypotheses. It should be noted that inter-device variability cannot be entirely ruled out as a cause for the low correlations found between accelerometers in Experiments 4 and 5. However, since there were strong correlations between activity monitors when they were on separate collars, inter-device variability is a less likely cause.

Finally, differences in activity data were found when the device was loosely zip-tied to the collar. When compared to the activity monitor that was threaded onto the collar using the provided metal loop, the zip-

Table 2 Summary of methods used in previous studies with the same activity monitor

Authors	Year	Method of Attachment	Leash Attachment	Device orientation
Yam, et al.	2011	Zip tied to collar	No leash	Not addressed
Hansen, et al.	2007	Various	No leash	Not addressed
Preston, et al.	2012	Various	Not addressed	Not addressed
Yashari, et al.	2015	Protective case on dedicated collar	No leash	Not addressed
Brown, et al.	2010	To collar – no details	Leash attached	Not addressed
Brown, et al.	2010	To collar – no details	Not addressed	Not addressed
Morrison, et al.	2014	To collar – no details	Not addressed	Not addressed
Morrison, et al.	2014	To collar – no details	Not addressed	Not addressed
Rialland, et al.	2012	To collar – no details	Not addressed	Not addressed
Rialland, et al.	2013	To collar – no details	Not addressed	Not addressed
Michel, et al.	2011	To collar – no details	Not addressed	Not addressed
Dow, et al.	2009	Not addressed	Not addressed	Not addressed

ted device was subjectively more movable. Logically, the low correlation between monitors in this experiment appears to be related to the activity monitor moving on the collar itself, artificially increasing activity counts. This occurred in spite of the zip ties being maximally tightened. These results are noteworthy because previous studies have reported zip-tying the activity monitor to the collar but did not detail the zip-tie method used [5]. Zip-tying the devices ideally should be avoided, however, given the small size of the metal loops (and thus the inability to fit thicker collars i.e. > 0.75 inches), care should be taken to ensure the same tightness if zip-ties are used to attach activity monitors to the collar.

The disconnect in previous publications [16, 18] between activity levels and other outcome measures such as gait analysis and owner questionnaires could be explained by the fact that activity levels represent a different measure of progression through a study period. While activity monitors may be addressing different aspects of outcome in these studies, the methods used to attach the activity monitors to the dogs cannot be ruled out as a source for the lack of correlation between activity levels and other measures utilized.

There were several limitations of this study, including the short duration that methods of attachment were evaluated for while walking. We chose this approach to eliminate variability in the type of activity, allowing for data acquisition in a controlled environment with a defined type of activity. Since activity monitors were directly compared for any given method of attachment, greater activity levels are likely only to further affect the results. Furthermore, given the large number of data points acquired, longer observation periods would be unlikely to change the results. It is also unknown how the varying activity levels of dogs in their daily lives would affect activity data. For instance, it is possible that the method of attachment would not have as large of an effect on a dog with a sedentary lifestyle. Further studies are necessary to characterize how the high and low extremes of activity level would impact activity data output amongst various methods of attachment. A second limitation of this study is that only one brand of accelerometer-based activity monitor was evaluated. Several other devices are available and it is unknown how the method of attachment would affect activity data from those monitors. However, until a similar evaluation of those devices is performed, we would suggest assuming that similar findings would apply to any activity monitor and the recommendations from this study should be followed regardless of which activity monitor is used. A final limitation of this study is the small sample size. However, in contrast to clinical studies evaluating naturally occurring disease, this study was designed to eliminate confounding factors and therefore a smaller sample size can be utilized to identify significant differences between groups. Additional studies with larger sample sizes that evaluate the identified factors in a clinical setting are necessary to further characterize accelerometer-based activity monitors as objective outcome measurement tools.

Conclusions

In conclusion, when utilizing accelerometers as a research tool care must be taken to clearly specify the method of attachment. Since retrieving data from the activity monitor utilized in this study requires removal of the device from the collar, the method of attachment should be recorded and kept consistent throughout the study period. Connecting a leash to the collar to which an activity monitor is attached should be avoided, as it is difficult to keep the amount of tension on the lead while walking consistent throughout a study. Lastly, data obtained when using the protective case should not be compared to data collected without the casing. Our results indicate that the protective casing considerably affects the activity data. As such it may be advisable to avoid use of any case entirely.

Endnotes

[1]Boots and Barkley® Core Fashion Collar, Size Medium, Target Brands, Inc., Minneapolis, MN

[2]Actical, Mini Mitter Company, Inc., Bend, Oregon

[3]Easy Walk Harness, PetSafe®, Radio Systems Corporation, Knoxville, Tennessee

[4]ActiReader, Mini Mitter Company, Inc., Bend, Oregon

[5]Excel, Microsoft Corporation, Redmond, Washington

[6]SPSS 22, IBM, Inc.

Abbreviations

CBPI: Canine Brief Pain Inventory; HCPI: Helsinki Chronic Pain Index

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Authors' contributions

KM carried out the activity monitoring experiments, data organization, and drafted the manuscript. AO participated in study design and helped draft the manuscript. CD participated in study design, performed statistical analysis and helped draft the manuscript. FD conceived of the study, participated in study design and coordination, and helped draft the manuscript. All authors read and approved of the final manuscript before its submission.

Competing interests

The authors declare that they have no competing interests.

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Anti-nausea effects and pharmacokinetics of ondansetron, maropitant and metoclopramide in a low-dose cisplatin model of nausea and vomiting in the dog

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Abstract

Background: Nausea is a subjective sensation which is difficult to measure in non-verbal species. The aims of this study were to determine the efficacy of three classes of antiemetic drugs in a novel low dose cisplatin model of nausea and vomiting and measure change in potential nausea biomarkers arginine vasopressin (AVP) and cortisol. A four period cross-over blinded study was conducted in eight healthy beagle dogs of both genders. Dogs were administered 18 mg/m^2 cisplatin intravenously, followed 45 min later by a 15 min infusion of either placebo (saline) or antiemetic treatment with ondansetron (0.5 mg/kg; 5-HT$_3$ antagonist), maropitant (1 mg/kg; NK$_1$ antagonist) or metoclopramide (0.5 mg/kg; D$_2$ antagonist). The number of vomits and nausea associated behaviours, scored on a visual analogue scale, were recorded every 15 min for 8 h following cisplatin administration. Plasma samples were collected to measure AVP, cortisol and antiemetic drug concentrations.

Results: The placebo treated group vomited an average number of 7 times (range 2–13). None of the dogs in either the ondansetron or maropitant treated groups vomited during the observation period. The onset of nausea-like behaviour in the placebo-treated group occurred at $t_{3.5h}$ and peaked at $t_{4.75h}$ with nausea behaviour score of 58.5 ± 4.6 mm. Ondansetron and maropitant reduced overall the area under the curve of nausea behaviour score by 90% and 25%, respectively. Metoclopramide had no effect on either vomiting or nausea.
Cisplatin-induced nausea and vomiting caused concomitant increases in AVP and cortisol. In the placebo-treated group, AVP and cortisol increased from $t_{2.5h}$, peaked at t_{5h} (11.3 ± 2.9 pmol L^{-1} and 334.0 ± 46.7 nmol/L, respectively) and returned to baseline by t_{8h}. AVP and cortisol increases were completely prevented by ondansetron and only partially by maropitant, while metoclopramide had no effect. The terminal half-lives (harmonic mean ± pseudo SD) for ondansetron, maropitant and metoclopramide were 1.21 ± 0.51, 5.62 ± 0.77 and 0.87 ± 0.17 h respectively.

Conclusions: 5-HT$_3$ receptor antagonist ondansetron demonstrates the greatest anti-emetic and anti-nausea efficacy of the three drugs. AVP and cortisol appear to be selective biomarkers of nausea rather than emesis, providing a means of objectively measuring of nausea in the dog.

Keywords: Nausea, Emesis, Cisplatin, Antiemetic, Maropitant, Ondansetron, Metoclopramide, Biomarker, Arginine vasopressin, Cortisol

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Background

Nausea is a subjective sensation induced by a variety of emetic stimuli and usually preceeds emesis. Nausea is a graded response with a dynamic threshold influenced by a variety of factors [1], unlike emesis, which is an all or nothing event occurring when emetic stimuli surpass the threshold required to activate the emetic reflex. Defining nausea in animals is inherently problematic due to its subjective nature and the inability of an animal to verbalise the sensation that they experience. Whether or not animals experience the sensation of nausea in the same way as people do is a contentious issue [2]. However, for the purposes of this article the term nausea will be used to denote the aversive state and prodromal response induced by the administration of a known emetogenic substance. Nausea-like behaviour refers to observable behaviours in animals that occur during the aversive state.

Antiemetic drugs work by blocking the emetic pathway at various points preventing the emetic reflex. Three major classes of anti-emetics are currently available for the treatment of chemotherapy-induced nausea and vomiting: 5-hydroxytryptamine$_3$ (5-HT$_3$), neurokinin$_1$ (NK$_1$) and dopamine$_2$ (D$_2$) receptor antagonists. All classes of antiemetic drugs have some anti-nausea efficacy [3–5] but are generally less efficacious for the treatment of nausea [6]. Human patients now report that nausea rather than emesis is the most distressing side effect of cancer chemotherapy [7]. The sensation of nausea stems from complex mechanisms involving cortical structures in the forebrain responsible for conscious perception, these mechanisms are thus far poorly characterised [8–10]. This has hindered the development of anti-nausea drugs.

Cisplatin is a platinum based cytotoxic drug used for the treatment of cancer [11]. It is highly emetogenic [12] and has been widely used experimentally to induce nausea and emesis in a variety of species including *Suncus murinus* [13], ferret [14] and the dog [15]. The low dose cisplatin model of nausea and emesis in the dog [16] induces significantly less emesis than previous models which utilise clinical doses of cisplatin [15]. The reduced emesis in the low dose model facilitates recognition of the behavioral signs of nausea and grading of its severity without the bias of frequent emetic events. Therefore, it is a good model for the assessment of the anti-nausea effects of currently available anti-emetic drugs and to relate the concentration of nausea biomarkers to the observed signs of nausea behaviour.

Potential nausea biomarkers, arginine vasopressin (AVP) and cortisol, have been identified as correlates of nausea behaviour in the dog [16]. Increased AVP has been positively correlated to self-reported nausea scores by volunteers, in which, motion sickness is induced [17, 18]. To date, these biomarkers have not been used in experimental intervention studies to assist in objective assessment of nausea.

The principal aim of the current study was to determine the relative efficacy of the representatives of three classes of antiemetic (ondansetron, maropitant, metoclopramide) in the prevention of nausea and emesis in the low dose cisplatin model.

Methods

Animals

Animal procedures undertaken were approved by the UK Home Office Animals (Scientific Procedures) Act 1986 (ASPA), Project license 70/7269 with ethical approval of the Royal Veterinary College (RVC) Ethics and Welfare Committee. Eight healthy neutered Beagle dogs (Marshall Farms, North Rose, NY, USA), four male and four females weighing from 6.5 to 11.5 kg, aged 2.5 years old at the start of the studies were used. Dogs were group-housed according to sex on a 12 h light/dark cycle and fed canine Lab diet 5007 (IPS Ltd., London, UK) once daily (100–200 g adjusted as needed to maintain ideal weight). Water was available ad libitum. The dogs were discharged from ASPA and re-homed as pets at the end of the study with the approval of the RVC's Named Veterinary Surgeon.

Experimental design

Dogs received cisplatin and antiemetic or placebo treatment in each of the 4 periods of the study. The 4 study periods were blocked by sex and by day with at least a 28 day wash out period being observed between doses of cisplatin. Antiemetic or placebo treatment was randomly allocated during period 1 and a Latin square design was used to determine treatment allocation in the remaining periods so that all dogs received each treatment over the course of the study.

Jugular vein catheter placement

One day prior to cisplatin administration, a double lumen jugular catheter was implanted under general anesthesia using the methods described in Kenward et al. [16].

Operator safety

Cisplatin was dispensed with the medical closed system (BD Phaseal, Oxford, UK) under a cytotoxic hood. Appropriate personal protective equipment (PPE) was worn by users when handling cisplatin and contaminated waste. Dogs were quarantined for 10 days following cisplatin treatment, PPE was worn to handle dogs and all waste was considered to be contaminated with cytotoxic material.

Fluid treatment

All dogs were given 0.9% saline and mannitol infusions prior to the administration of cisplatin and an hourly saline bolus post cisplatin through the first lumen of the jugular catheter as described in Kenward et al. [16] to reduce the nephrotoxic effects of cisplatin (Fig. 1).

Cisplatin administration

The time of initiation of cisplatin infusion was measured in hours and defined as t_{0h} (Fig. 1). At t_{0h}, the dogs received 18 mg/m^2 of cisplatin (Hospira, Leamington Spa, UK). Dogs were weighed on the day preceding the injection and body surface area (m^2) calculated using the formula:

$$Dose\ (m^2) = \frac{k.W^{2/3}}{100}$$

where constant k = 10.1 and W is the weight of the dog in kilograms [19].

The appropriate volume of stock cisplatin solution (1 mg/mL) for each dog was diluted in 0.9% saline to produce a standard volume of 40 mL. The diluted cisplatin solution was administered through the second lumen of the jugular catheter using automatic dispensing syringe at a flow rate of 2.0 mL/min.

Antiemetic administration

Anti-emetic or placebo administration was completed at t_{1h} following cisplatin treatment. Depending on treatment allocation from the experimental design, dogs received one of the following: 0.5 mg/kg ondansetron (Zofran®, GSK, Brentford, UK), 1 mg/kg maropitant (Cerenia®, Zoetis, Florham Park, NJ, USA), 0.5 mg/kg metoclopramide (Emeprid®, Ceva Santé Animale, Amersham, UK) or 0.9% saline as placebo. All treatments were made up to a standard volume of 15 mL using 0.9% saline and were administered intravenously through the second lumen of the jugular catheter at a flow rate of 1.0 mL/min.

Behavioural assessment

Prior to the commencement of studies an acclimatization period of 1 month was allowed for the dogs to become familiar with the experimental context. During this period, the observer became accustomed to each individual dog's normal behaviour, which was used as a baseline reference for behavioural observations. During the 28-day washout interval between the study periods, the dogs were placed in the experimental context weekly to prevent the development of a conditioned response.

Observations of behaviours suggestive of a nausea-like state and the number of vomits were recorded for 7 h following cisplatin treatment. Nausea-like behaviour was recorded by a single trained observer, who was blinded to the treatment the dogs had received. A composite 'nausea' score was recorded using a visual analogue scale (VAS) [15]. The observer made a judgment on the severity of the dog's nausea-like behaviours based on the presence and frequency of one or more of the following during a 15 min time bin; salivation, lip licking, lethargy, restlessness or turning/circling behaviour signaling that vomiting is imminent. A score of 0 mm indicated 'No 'Nausea" and a score of 100 mm indicated 'Worst

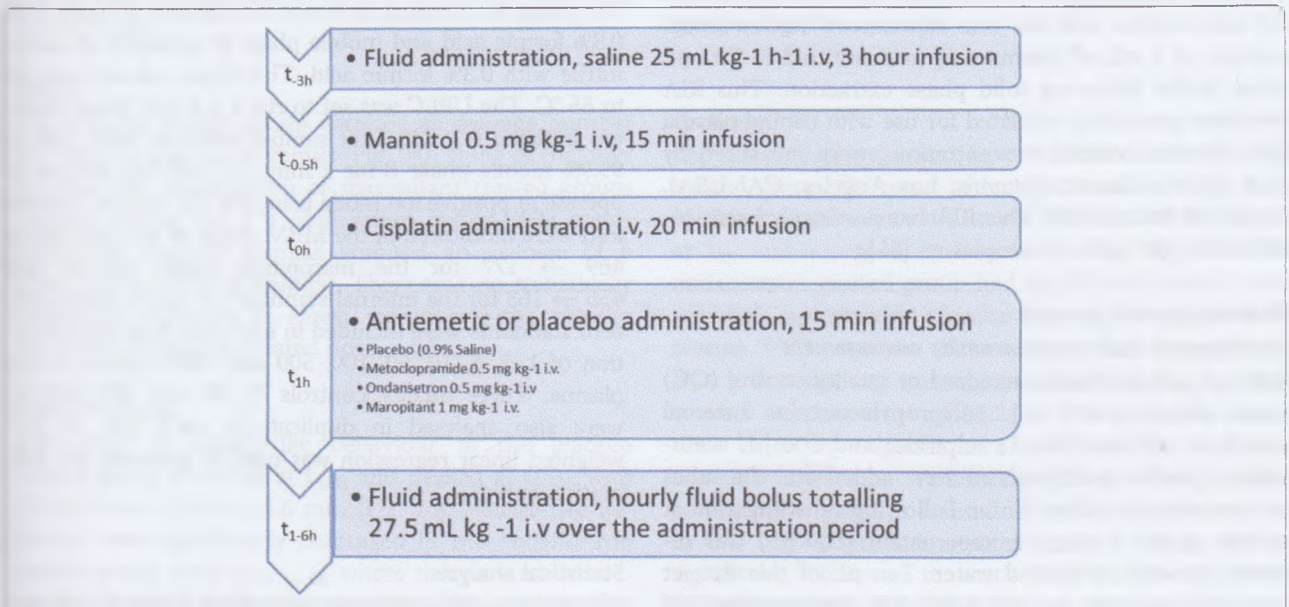

Fig. 1 Fluid and drug administration protocol

possible 'Nausea". This method of behavioural assessment in dogs was based on a 'nausea' visual analogue scale described by de la Puente Redondo et al. [15] and was validated 'in-house', for a cisplatin-induced nausea and vomiting, by the authors in Kenward et al. [16].

Blood sampling

Venous blood samples were collected from the jugular catheter for biomarker analysis at t = −3 (baseline), 0, 2.5, 4, 4.5, 5, 5.5, 6, 7, 8 and 24 h following cisplatin administration. Additional samples were collected for pharmacokinetic analysis of the antiemetic drugs at t = 1.25, 1.5, 1.75, 2.25, 2.75, 3.25, 4.25, 6.25, 8.25 and 24.25 h following cisplatin administration. Approximately 5 mL of blood was collected for biomarker analysis and 0.5 mL for PK analysis at each time point, giving a maximum of 60 mL collected per dog. Blood was collected into EDTA coated tubes or Lithium Heparin coated tubes as appropriate for subsequent assays. In agreement with ASPA regulations, a maximum of 15% blood volume was withdrawn in any 28 day period, with no more the 10% withdrawn in any 24 h period. Blood sample tubes were placed in an ice bath for a maximum of 10 min before centrifugation at 4000 x g for 15 min. Plasma samples were aliquoted into individual tubes, A protease inhibitor cocktail (15 μM; Sigma Aldrich, UK) was added to tubes containing plasma for vasopressin assay. All samples were snap frozen on dry ice then stored at −80 °C prior to analysis.

Biomarker measurement

Vasopressin was extracted from the plasma and the concentrations were measured by radioimmunoassay (RIA) (RB-319, Eurodiagnostica AB, Malmö, Sweden), as per kit instructions with the one adjustment that an initial sample of 1 mL of plasma was reconstituted in 700 μL assay buffer following solid phase extraction. This RIA has been previously validated for use with canine plasma [20]. Plasma cortisol concentrations were measured by RIA (Coat-a-Count®, Siemens, Los Angeles, CA, USA), as per kit instructions. This RIA has previously been validated for use with canine plasma [21].

Pharmacokinetic analysis

Ondansetron and metoclopramide measurement

Fifty μL sample, blank, standard or quality control (QC) were added to a 2 mL polypropylene tube. Internal standard solution (10 μL; sulpiride) and 200 μL acetonitrile (protein precipitation) were added and the tubes were vortex mixed for 5 min. Following centrifugation at 13400 xg for 2 min, the supernatant (100 μL) was diluted 1:1 with de-ionised water. Ten μL of this extract were injected onto the HPLC/MS/MS. Ondansetron and metoclopramide concentrations were measured by HPLC/MS/MS using a 10 cm × 4.6 mm SUPELCOSIL™ LC-Si HPLC Column maintained at 50 °C. This was coupled with electrospray ionisation (ESI) and tandem mass spectrometry (API 4000, Applied Biosystems). The mobile phase consisted of acetonitrile/10 mM ammonium formate (80/20 *v*/v) delivered at 1 mL/min. Positive ions were monitored in the MRM mode with m/z transitions 300 → 227 for metoclopramide, 294 → 212 for ondansetron and 342 → 112 for internal standard sulpiride. Six non-zero standards were included in each run, final concentrations of 2.5, 10, 25, 50, 75 and 100 ng/mL in plasma. Three quality controls (15, 30 and 75 ng/mL) were also analysed in duplicate in each run. A $1/x^2$ weighted linear regression was used to generate the calibration curve for both drugs.

Maropitant measurement

Hundred μL sample, blank, standard or quality control (QC) were added to a 96 well polypropylene plate containing 400 μL acetonitrile (protein precipitation) with 5 ng/mL of internal standard CJ-12191. The plate was sealed and centrifuged for 15 min at 1700 x *g* to pellet precipitated proteins. Ten μL of supernatant was added to a 96 well injection plate containing 790 μL water/acetonitrile with 0.1% formic acid (50/50, *v*/v). Maropitant concentrations were measured by ultra-performance liquid chromatography (UPLC) (Waters Acquity, Milford, MA, USA) using Waters ACQUITY column BEH C 1.7 μm, 2.1 × 50 mm, equipped with VanGaurd ™ Pre-Column. This was coupled with electrospray ionization (ESI) and tandem mass spectrometry (MS/MS) (API 4000, Applied biosystems/MDS Sciex, Frarmingham, MA, USA). The range of the assay is 1–1000 ng/mL. The mobile phase A consisted of 5 mM ammonium formate with 0.3% formic acid and mobile phase B consisted of acetonitrile with 0.3% formic acid. The target column was set to 55 °C. The UPLC was set to run a 1.8 min linear gradient initiated 0.2 min after sample injection from 10% to 99.9% mobile phase B for 2 mins. LC-MS/MS was set to operate in positive ion mode using the ESI source. Positive ions were monitored in the MRM mode of m/z transitions 469 → 177 for the maropitant target analyte and 455 → 163 for the internal standard CJ-12191. Eight non-zero standards were included in each run, final concentration of 1, 2, 5, 10, 50, 100, 500 and 1000 ng/mL in dog plasma. Three quality controls (3, 30 and 800 ng/mL) were also analysed in duplicate in each run. A $1/x^2$ weighted linear regression was used to generate the calibration curve.

Statistical analysis

All statistical analyses were carried out using PASW statistics 18 v 18.0.0 (SPSS: An IBM company, Chicago, IL,

USA). All data are presented as mean ± SEM and significance levels were set at $P \leq 0.05$.

After checking normality of the distribution, correlation analyses were carried out between nausea-like behaviour and change in biomarkers, AVP and cortisol (Pearson's coefficient). Time courses of nausea-like behaviour and change in biomarkers were analysed using a linear mixed-effect model with 1st order autoregressive covariance structure. The statistical model included the fixed effects of treatment group, time and group*time interaction, the repeated effects of time and the random effect of subject. A least significant difference *post-hoc* pairwise comparison of anti-emetic treatment was carried out at each time point to determine if there was a significant interaction between treatment and time. The area under the curve (AUC) was calculated for time-course of change of nausea-like behaviour and biomarkers, AVP and cortisol, by determining the trapezoidal area under the curve. AUCs and the difference in the number of vomits for each antiemetic treatment group were compared using one-way ANOVA with Tukeys post hoc comparison.

Pharmacokinetic parameters and compartmental modelling was carried out using WinNonlin professional software (WinNonlin, Version 5.2, Pharsight Corp, Mountain View, CA, USA).

Results

The repeated administration of cisplatin throughout the study was well tolerated as previously reported in Kenward et al. [16]. Each treatment group consisted of $n = 8$ except for the metoclopramide group where $n = 7$ as one dog was excluded from the last period due to the development of atopic dermatitis unrelated to the study.

Emesis

The placebo treated group vomited an average number of 7 times (range 2–13 vomits; Fig. 2a). None of the dogs in either the ondansetron or maropitant treated groups vomited during the 8 h observation period. The metoclopramide treated animals vomited an average of 6 times (range of 3–10 vomits). There was no statistically significant reduction in emesis comparing the placebo and metoclopramide treated dogs.

Signs of nausea

The onset of nausea-like behaviour in the placebo treated group occurred at $t_{3.5h}$ and peaked at $t_{4.75h}$ with a VAS value of 58.5 ± 4.6 mm (Fig. 2c). Nausea-like behaviour was significantly decreased in the ondansetron treated group from $t_{3.75\ to\ 6h}$ where the peak nausea response was VAS 11.9 ± 7.0 mm at $t_{4.5h}$ (Fig. 2d). In both the maropitant and metoclopramide treatment groups, the onset of nausea-like behaviour was delayed. VAS scores were significantly reduced compared to placebo at $t_{3.75\text{-}4h}$ and $t_{4.5}$ h in the maropitant treated group and at $t_{3.75\text{-}4h}$ in the metoclopramide treated group. In the maropitant treatment group, the peak nausea response was 49.8 ± 7.4 mm occurring at $t_{4.75h}$ (Fig. 2e). The peak nausea response for the metoclopramide treatment group was significantly increased from placebo where the VAS score was 72 ± 6.9 mm at $t_{4.5h}$ (Fig. 2f). The AUC for nausea-like behaviour was only significantly reduced in the ondansetron treated group compared placebo, a 90% reduction (Fig. 2b).

Biomarkers

Arginine vasopressin

In the placebo treated group the onset of AVP increase occurred at $t_{2.5h}$ and peaked at t_{5h} with a value of 11.35 ± 2.92 pmol/L (Fig. 3). Following cisplatin administration, the AVP concentration in the ondansetron treated group did not exceed the baseline plasma AVP concentration (1.11 ± 0.84 pmol/L) at any time in the 8 h following cisplatin administration and was significantly reduced compared to placebo from t_{4h} to t_{8h}. Plasma AVP was significantly decreased in the maropitant treated group compared to placebo at $t_{4.5h}$ and $t_{5.5h}$ but the peak of 9.79 ± 3.39 pmol/L was not significantly different from placebo. No significant difference was detected between the placebo and the metoclopramide treated group at any time point during the study. The AVP AUC of the ondansetron treated group was significantly reduced compared to placebo ($P < 0.001$). There was no significant difference in AUC between placebo and either the maropitant or the metoclopramide treated groups (Fig. 3).

Cortisol

Plasma cortisol increased from baseline in the placebo treated group, the onset of increase occurred from $t_{2.5h}$, reaching a peak concentration of 334.05 ± 46.71 nmol/L at t_{5h} and returned to baseline by t_{8h} (Fig. 4). The ondansetron treated group had significantly lower cortisol levels compared to placebo from t_{4h} to $t_{5.5h}$. Like the plasma AVP response, plasma cortisol of the ondansetron group did not exceed mean baseline (t_{-3h}) plasma cortisol concentration of 87.21 ± 29.14 nmol/L at any time following cisplatin administration. Cortisol concentrations were not statistically different from placebo at any time point measured in either the maropitant or the metoclopramide treated groups. There was no significant difference in cortisol AUC for any of the anti-emetic drug treatment groups compared to placebo (Fig. 4).

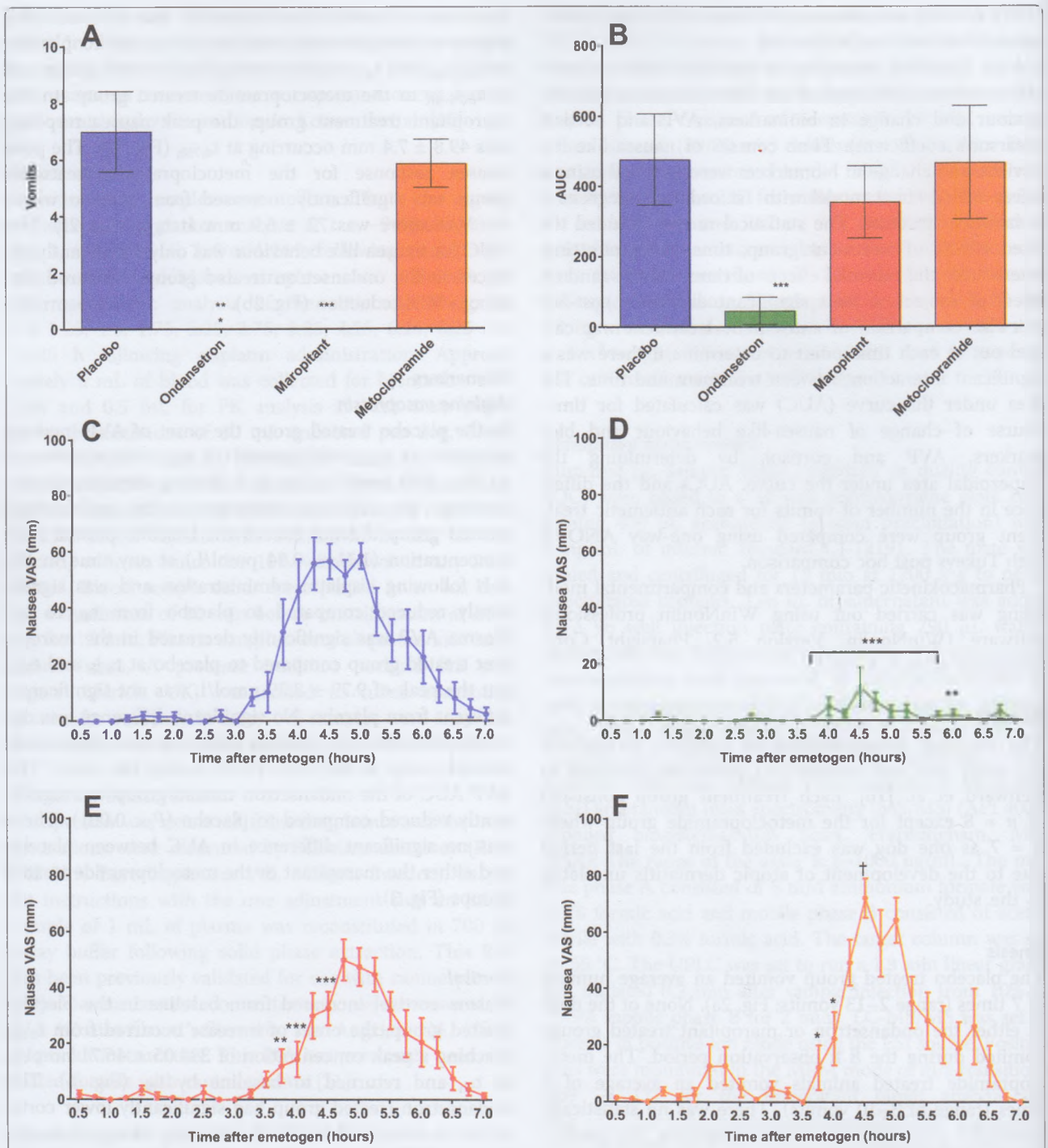

Fig. 2 a-f Emesis and Nausea behaviour following 18 mg m^{-2} cisplatin i.v. The number of vomits (**a**) and area under the curve of nausea behaviour (**b**) for all 3 anti-emetic drugs compared to placebo. Timecourse of nausea-like behaviour (VAS) following 18 mg m^{-2} cisplatin i.v. for each treatment groups; placebo (**c**), ondansetron 0.5 mg kg^{-1} (**d**), maropitant 1 mg kg^{-1} (**e**), metoclopramide 0.5 mg kg^{-1} (**f**). Values presented as mean ± SEM, $n = 8$ per group, except metoclopramide group where $n = 7$. Significant decrease of antiemetic treated groups compared to placebo: mixed linear model (**$P < 0.01$, *** $P < 0.001$), Significant increase in nausea behaviour between placebo and anti-emetic treated groups: mixed linear model (†$P < 0.05$). Anti-emetic treated groups compared to placebo; Area under curve and number of vomits, ANOVA (*$P < 0.05$, ***$P < 0.001$)

Biomarkers vs nausea-like behaviour correlation

There was a weak significant correlation between AVP and VAS ($P = 0.0065$, $R^2 = 0.1362$) and between Cortisol and VAS ($P < 0.0001$ and $R^2 = 0.2699$). The relationship between these variables and VAS was distorted because of the time lag between the rise in VAS and

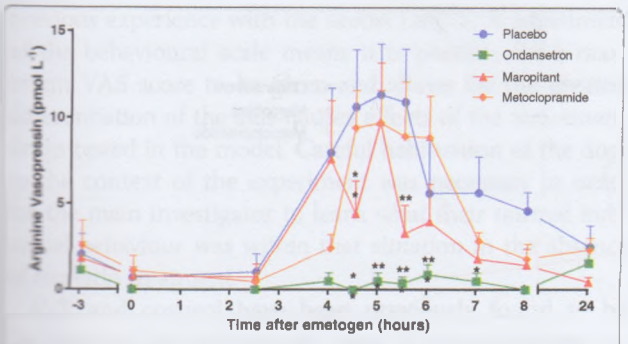

Fig. 3 Plasma arginine vasopressin following 18 mg m^{-2} cisplatin i.v. Timecourse of change in plasma AVP following 18 mg m^{-2} cisplatin i.v. comparing placebo to each of the antiemetic treatment groups. Values presented as mean + SEM, $n = 8$ per group, except metoclopramide group where $n = 7$. Significant differences between placebo and anti-emetic treated groups: mixed linear model $^{*}P < 0.01$, $^{***}P < 0.001$)

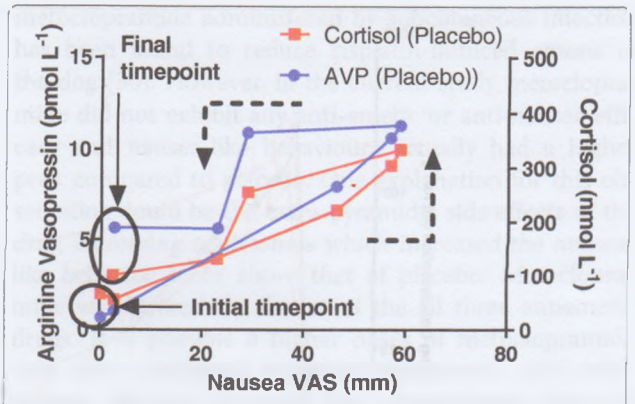

Fig. 5 Negative Hysteresis relationship for Cortisol and arginine vasopressin (AVP) versus nausea-like behaviour (group average, $n = 8$ dogs) following 18 mg m^{-2} cisplatin i.v. The time courses of changes in both biomarkers against VAS are indicated by the dotted black arrows (anticlockwise.) AVP rises with VAS scores but persist to high level when the nausea score decreases (hysteresis loop suggesting a time delay), whereas cortisol follows nausea scores changes more closely in time

The rise of the biomarkers and can be better visualised with a hysteresis plot (Fig. 5.). AVP rose with VAS scores but persisted to high level when the nausea score decreased (hysteresis loop suggesting a time delay), whereas cortisol followed nausea scores changes more closely in time.

Pharmacokinetics

Goodness of fit of the standard curves was $R = 0.9986$, 0.9863 and 0.9995 for ondansetron, maropitant and metoclopramide, respectively. The accuracy assays ranged between 101.3% to 108.6% for ondansetron, 92.5% to 119% for maropitant and 89.7% to 96.3% for metoclopramide,. Plasma concentration-time profiles are presented in Fig. 6. Disposition for all 3 antiemetic drugs was best described by a two-compartment model. Maximal concentration was at the completion of the 15 min intravenous infusion (t_{1h}). All PK parameters are summarised in Table 1.

Ondansetron concentrations in plasma were below the lower limit of quantification in 3 of 8 dogs at t_{7h} and in all animals by t_{24h}. Maropitant was still detectable in the plasma of all dogs at t_{24h}. Metoclopramide concentrations in plasma fell below the lower limit of quantification in 3 of 7 dogs at t_{5h} and in all animals at t_{7h}.

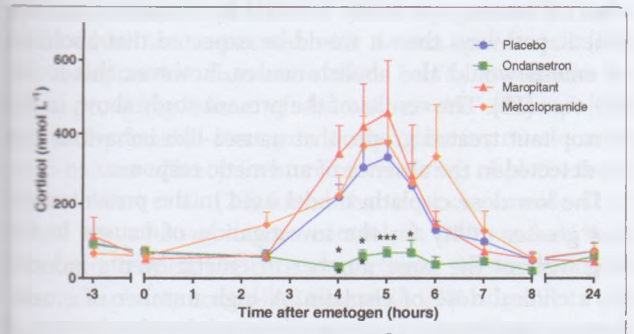

Fig. 4 Plasma cortisol following 18 mg m^{-2} cisplatin i.v. Timecourse of change in plasma cortisol following 18 mg m^{-2} cisplatin i.v. comparing placebo to each of the antiemetic treatment groups. Values presented as mean + SEM, $n = 8$ per group, except metoclopramide group where $n = 7$. Significant differences between placebo and anti-emetic treated groups: mixed linear model $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$)

Discussion

The present study, using a low dose model of cisplatin to induce nausea and vomiting, has demonstrated differential relative anti-emetic and anti-nausea efficacy within three classes of anti-emetic drugs when used at dose rates previously shown to inhibit emesis in the dog. Ondansetron inhibited both emesis and all behavioural signs of nausea completely. Maropitant inhibited emesis completely but only partially reduced the behavioural signs of nausea at the early time points. Metoclopramide had no significant effects on the number of episodes of vomiting and very little effect on the behavioural signs of nausea. These results support the hypothesis that emesis is easier to prevent than nausea by reducing the neuronal signals to below the threshold required to trigger emesis. By contrast, nausea, being a graded phenomenon, persisted in the presence of concentrations of maropitant which prevented dogs vomiting. The objective biomarkers of nausea, plasma AVP and cortisol identified by our previous characterisation of this low dose cisplatin model [16] add weight to the conclusions made based on subjective behavioural observations of nausea.

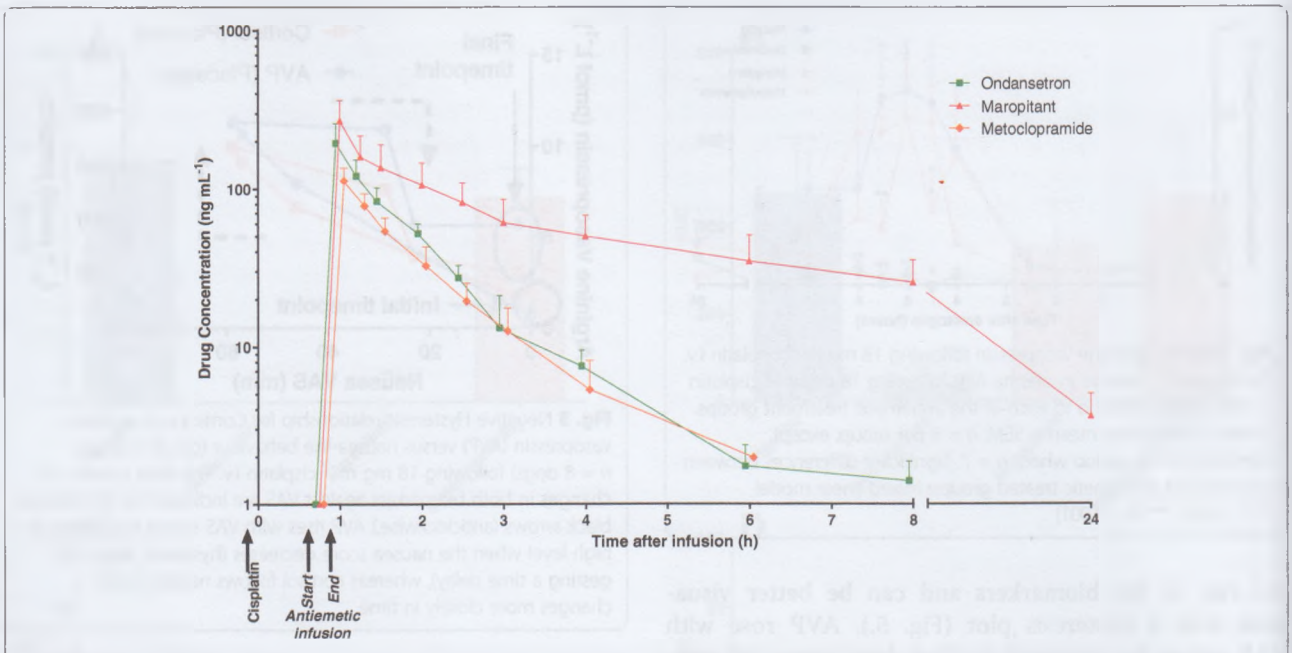

Fig. 6 Plasma concentration-time curve for the 3 antiemetic treatments. Timecourse of change in plasma concentration of the maropirant, metoclopramide and ondansetron after intravenous injection of 1 mg kg^{-1}, 0.5 mg kg^{-1} and 0.5 mg kg^{-1}, respectively. Values presented as mean + SEM, $n = 8$ per group, except metoclopramide group where $n = 7$

Cisplatin-induced nausea and vomiting has a biphasic time-course in human subjects. The 'acute' phase of nausea and vomiting occurs in the first 24 h following treatment. Nausea and vomiting occurring more than 24 h following treatment is referred to as the 'delayed phase' and can persist for 3–5 days [22, 23]. The present study focused on the initial 'acute' phase of cisplatin-induced nausea and vomiting which is thought to be predominantly peripherally mediated. Cisplatin causes the release of 5-HT from the gut which stimulates abdominal vagal afferent neurons that project to the 'vomiting centre' in the brainstem [22]. Cisplatin emesis can be abolished in dogs by abdominal vagotomy or the systemic administration of 5-HT_3 antagonists but not by the central administration of 5-HT_3 antagonists into the cerebral ventricles [24]. The 'delayed' phase is mediated mainly by a central mechanism of action. The area postrema (commonly referred to as the chemoreceptor trigger zone) located in the brainstem is thought to be stimulated by cisplatin, its metabolites, or gut peptides released in response to the effects of cisplatin on the intestine [22]. Ablation of the area postrema in ferrets abolished the delayed emetic response to cisplatin but bilateral vagotomy did not [23].

Table 1 Pharmacokinetic parameters for metoclopramide, ondansetron and maropitant after IV administration

Parameter	Units	Ondansetron ($n = 8$)	Maropitant ($n = 8$)	Metoclopramide ($n = 7$)
C_{max}	µg/L	186.8 ± 60.6	247.0 ± 86.2	113.2 ± 22.4
$t_{1/2}$	h	1.21 ± 0.51	5.62 ± 0.77	0.87 ± 0.17
$AUC_{0\text{-last}}$	µg/L/h	176.4 ± 32.6	705.2 ± 236	111.9 ± 27.2
Cl	L/kg/h	2.90 ± 0.43	1.57 ± 0.53	4.72 ± 1.25
MRT	h	1.15 ± 0.43	6.68 ± 1.30	1.04 ± 0.16

Legend: All results are presented as the arithmetic mean ± SD except $t_{1/2}$ which is presents as the harmonic mean ± Pseudo SD

Nausea, unlike emesis, is a multi-dimensional experience incorporating emotional and affective components in addition to the physiological response. The sensation of nausea is thought to arise from activation of cortical structures involved in conscious perception [9, 25, 26]. The mechanisms by which cisplatin induces nausea are less clear. If nausea was solely induced by stimulation of the emetic pathways then it would be expected that abolition of emesis would also abolish nausea, however, this is not the case [18]. The results of the present study show, in the maropitant treated group, that nausea-like behaviour can be detected in the absence of an emetic response.

The low dose cisplatin model used in the present study has greater utility for the investigation of nausea in the dog without the large number of emetic events induced by a clinical dose of cisplatin. A high number of emetic events may bias the observer when judging the severity of nausea as a function of the emesis observed rather than as a distinct sensation with specific associated behaviours, which may be obscured by frequent vomiting and retching. The behavioural assessment in the present study judged the maximum VAS score as the 'worst possible nausea' for the low dose model of cisplatin gained from

previous experience with the model [16]. This adjustment of the behavioural scale means it is possible for a maximum VAS score to be given and allows for the greatest differentiation of the anti-nausea effects of the anti-emetic drugs tested in the model. Careful habituation of the dogs to the context of the experiment was necessary in order for the main investigator to learn what their normal individual behaviour was within that situation in the absence of cisplatin treatment.

AVP and cortisol have been previously found to be correlates of the behavioural signs of nausea induced by cisplatin [16] but it was not clear if the emetic response also contributed to the change in these biomarkers. The results of the present study support AVP and cortisol as specific biomarkers of nausea rather than emesis. Complete inhibition of the behavioural signs of nausea in the ondansetron treated group results in reduction of AVP and cortisol to baseline levels whereas complete inhibition of emesis and only partial inhibition of the behavioural aspects of the nausea in the maropitant treated group led to only partial inhibition of AVP and no inhibition of cortisol. AVP appears to provide the more sensitive marker of nausea-like behaviour as partial inhibition of nausea-like behaviour by maropitant was identified through a significantly reduced AVP compared to placebo at $t_{4.5h}$, which was not evident in the cortisol response. The relationship between these nausea biomarkers and the nausea-like behavioural response requires further characterisation in a study using a range of doses of ondansetron and maropitant that describe the full concentration anti-nausea effect relationship of these two drugs more completely than is possible from the data generated by the present study.

D_2 receptor antagonists, such as metoclopramide, have both central and peripheral mechanisms. They act centrally on D_2 receptors in emetic brainstem regions, such as the area postrema and dorsal vagal complex [27]. Peripherally, they are prokinetic, resolving gastric dysrhythmias which are associated with nausea and emesis [18]. Metoclopramide also has a weak 5-HT_3 antagonist action providing some of its anti-emetic efficacy [28]. Metoclopramide has previously demonstrated some anti-nausea efficacy in humans receiving cisplatin treatment. Allan et al. [29] found that metoclopramide abolished or achieved major control in 26% and 46% of cisplatin treated patients respectively. Metoclopramide also completely controlled nausea in 24% of patients and achieved major control of nausea in 47% of patients; this was increased to 32% and 62% respectively when combined with dexamethasone [29]. Metoclopramide was also found to significantly reduce the duration of nausea induced by cisplatin chemotherapy [3]. Metoclopramide is used frequently in veterinary medicine to control nausea and vomiting. A dose of 1–3 mg/kg of metoclopramide administered by subcutaneous injection has been found to reduce cisplatin-induced emesis in the dog [30]. However, in the current study metoclopramide did not exhibit any anti-emetic or anti-nausea efficacy and nausea-like behaviours actually had a higher peak compared to placebo. One explanation for this observation could be the extra-pyramidal side effects of the drug increasing restlessness which increased the nausea-like behavior score above that of placebo. Metoclopramide was cleared the fastest of the all three antiemetic drugs. It is possible a higher doses of metoclopramide may have produced a greater anti-emetic and anti-nausea efficacy, however the administered dose of 0.5 mg/kg was at the highest end of the recommended dose for dogs which is 0.25–0.5 mg/kg every 6–8 h [31] and worsening extra-pyamidal side effects are seen in dogs given high doses of metoclopramide [32].

Ondansetron was the first-in-class 5-HT_3 antagonist acting via both central and peripheral mechanisms. Peripherally, 5-HT_3 antagonists block the activation of abdominal vagal afferents by emetic stimuli in the gastrointestinal tract [33]. Centrally, blockade of 5-HT_3 receptors on the terminals of vagal inputs into emetic brain stem regions prevents emetic stimuli from reaching the vomiting centre [28]. Ondansetron significantly reduced the number of episodes of emesis, increased the latency to emesis and decreased nausea VAS scores compared to placebo in patients receiving cisplatin chemotherapy [4]. The level of control of nausea and emesis was significantly greater with ondansetron compared to metoclopramide and ondansetron treatment was preferred by patients [34, 35]. Ondansetron provided greatest efficacy in the control of acute nausea following cisplatin treatment, whereas metoclopramide was found to be significantly more efficacious at controlling delayed nausea [34]. The combination of ondansetron with dexamethasone further improved the level of control of nausea [36, 37]. Ondansetron also exhibits anti-emetic and anti-nausea efficacy in animal models of nausea and vomiting. Ondansetron significantly inhibits emesis induced by cisplatin in ferrets [38] and by methotrexate in dogs [39]. In a lycorine model of nausea and vomiting in beagle dogs, ondansetron significantly reduced emesis and also exhibited significant anti-nausea activity [40].

NK_1 antagonists are the most recent class of anti-emetic drugs, aprepitant being approved by the FDA in 2003 for use in humans and maropitant being approved in 2007 for use in the dog. The anti-emetic properties are thought to occur as a result of the blockade of NK_1 receptors in emetic brainstem regions, including the area postrema and the nucleus tractus solitarius [41, 42]. The addition of aprepitant to standard antiemetic therapy with dexamethasone and a 5-HT_3 antagonist improved the control of chemotherapy-induced nausea and

vomiting especially in the delayed phase. Aprepitant significantly reduced emesis overall and in both the acute and delayed phases [43–45]. Nausea was significantly reduced overall and in the delayed phase but not the acute phase [43, 45]. In a clinical dose (70 mg/m^2) cisplatin model of nausea and vomiting in the dog, maropitant significantly reduced emesis and had anti-nausea efficacy suppressing nausea-like behaviours across the full time-course and significantly decreasing the peak nausea response [15]. In the present study, maropitant exhibited less anti-nausea efficacy than reported by de la Puente Redondo et al. [15]. It is possible that maropitant may have greater efficacy against the more severe nausea induced by the higher dose of cisplatin. For this explanation to be correct would require severe nausea to be induced by different mechanisms at higher doses of cisplatin which seems unlikely. An alternative explanation is that the significant reduction of emesis induced by maropitant treatment may have caused a bias towards reduced nausea scores in the high dose cisplatin study, a bias that is not so much of a problem in the low dose cisplatin model used in the present study. Another difference between the present study and that of de la Puente Redondo et al. [15] is the route of administration of maropitant. In the present study maropitant was administered by i.v. infusion whereas it was given subcutaneously in the study by de la Puente Redondo et al. [15]. The alternative route of administration used in the present study leads to a higher initial concentration of maropitant and a shorter half-life compared to s.c. administration. Maropitant plasma concentrations could have been at sub-therapeutic concentrations during the nausea response in the present study. However, this is improbable as an antiemetic and anti-nausea effect demonstrated 19 h following administration 1 mg/kg maropitant s.c. [15] when plasma concentrations would certainly be lower than those during the nausea response in the present study.

The results of the present study suggest that both 5-HT_3 and NK_1 receptors are an integral part of the emetic pathway activated by cisplatin which results in activation of the emetic reflex. The sensation of nausea is produced in cortical forebrain regions [46], increased activity was recorded in the left amygdala, the ventral putamen and the putative locus coeruleus [47]. Nausiogenic signals travel from the vomiting centre via rostrally projecting pathways to the forebrain and nuclei controlling the physiological response to nausea sensation (e.g. salivating, restlessness). The ability of ondansetron to reduce cisplatin-induced nausea would suggest that 5-HT_3 receptors have a role in transmitting nausea stimuli, either from the brainstem, the periphery or both, whereas NK_1 receptors are limited to a central emetic triggering mechanism.

Conclusions

In a low dose cisplatin model of nausea and emesis in the dog, NK_1 antagonist demonstrated good anti-emetic activity but limited anti-nausea effect. The 5-HT_3receptor antagonist ondansetron was most effective at treating both cisplatin-induced nausea and vomiting with associated reductions in nausea biomarkers AVP and cortisol. Further study using escalating doses of anti-emetics as interventions to inhibit nausea induced by different stimuli would be beneficial to determine the full PK/PD relationship of the anti-nausea effects of anti-emetics and explore the utility of nausea biomarkers in studying the pathways that can give rise to nausea.

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Authors' contributions
HK, LP and JE designed the research study. HK, LP and TL preformed the research. HK and LP analysed the data. HK, LP and JE wrote the paper. All authors read and approved the final manuscript.

Consent for publication
Not applicable.

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In vitro properties of concentrated canine platelets stored in two additive solutions

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Abstract

Background: Platelet transfusion therapy poses many challenges in veterinary clinical practice. Lack of readily available blood donors, short shelf-life, and inability to administer a sufficient number of platelets to meet a dog's transfusion need are the major difficulties encountered. Platelet additive solutions are already in use at American and European human blood banks, showing to be a realistic alternative. This study compares the in vitro platelet function in plasma, Composol, or SSP+ during storage for 13 days. Platelet rich plasma-platelet concentrate with 35% plasma and 65% platelet additive solutions (Composol or SSP+) and a control group (100% plasma) were prepared. Swirling, platelet count, blood gases, metabolic variables, platelet activation markers, and apoptosis markers were analyzed on days 1, 5, 9 and 13.

Results: Swirling was well preserved and pH was acceptable (> 6.2) during storage for all platelet additive solutions units until day 9. SSP + units showed more stable pH and metabolic variables until day 13. Platelets in plasma showed higher glucose consumption than in Composol or in SSP+. The platelet additive solutions units showed better platelet metabolism maintenance, reduced glucose consumption and lactate production. The apoptotic markers were still low for 9 days in platelet concentrates with platelet additive solutions, suggesting the possibility to extend the shelf life with the use of SSP+ or Composol.

Conclusions: Our findings suggest that the uses of Composol and SSP+ in canine platelet concentrates are potential alternatives in veterinary blood banks.

Keywords: Composol, Platelet storage lesion, SSP +, Storage solutions, Veterinary transfusion medicine

Background

One of the goals of transfusion medicine is to assure the production of quality blood components. The platelet concentrate (PC) availability is limited due to its short-term storage. During storage, platelets suffer structural and biochemical changes that are collectively called platelet storage lesions (PSL) [1]. Extending the shelf-life has effects on the quality of platelets which have been studied in PC stored for more than 5 days. For several reasons, storing platelets in platelet additive solutions (PAS) is turning into a routine practice, and it is already standard in human blood banks in Europe and The United States [2].

PAS are isotonic crystalloid media containing citrate as anticoagulant and acetate as fuel for aerobic metabolism. Initially, the main motivation to use PAS was to increase the availability of plasma for the fractioning and production of derived components. However, other advantages were observed, such as being a sterile environment free of pathogens and the standardized composition in comparison to plasma from donors [3–5].

Several PAS formulations are being tested since 1980. In vivo studies with the most recently developed solutions have shown good results of platelet increment and recovery as well as the reduced occurrence of transfusion reactions in human patients [6–8].

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Studies in human blood banks have already tested the platelet metabolism in vitro using PAS for periods exceeding 12 days [9, 10]. In some countries in Europe, the storage timing of PC was extended to 7 days with the use of PAS [2]. There are already four PAS generations, Composol (PAS-D) and SSP+ (PAS-E), tested in this study, are from the third generation and are used to preserve PC in European blood banks [3, 6, 11].

The search for new additive solutions for PC preservation has always been a concern in human medicine, and nowadays it is also of interest for the veterinary medicine. The biochemical evaluation during canine PC storage with different PAS represents some practical information for veterinary medicine. Canine platelet metabolism has similar characteristics to the human, and the PC storage time is also a difficulty at the veterinary blood banks. The quality control requirements of canine PC are based on human models. Therefore, it is believed that the results concerning the viability and PSL should be similar to the already reported in vitro tests done with human PC.

This study aims to compare the parameters of in vitro platelet metabolism from concentrates of canine platelets stored in plasma and in two PAS.

Methods

A volume of 450 mL whole blood was collected in blood bag with CPD[1] from healthy canine donors, weighing over 28 kg. Physical examination, CBC, chemistry profile, and infectious disease screening were performed for each dog. The animals were privately owned; all owners provided their consent for their dogs to be used in this study in accordance with bioethics concepts applied to animal research. This study was approved by the Ethics Committee on Animal Use from the Federal University of Rio Grande do Sul (Approval protocol #20528).

The experiment was carried out in three groups (Treatment 1: 100% plasma (control) $n = 13$; Treatment 2: SSP+[2] $n = 13$; Treatment 3: Composol[3] $n = 14$), in a total of 40 blood bags.

PC preparation

After collection, the bags were left undisturbed at room temperature for one hour and then were processed to obtain the PC through the platelet rich plasma method (PRP). The first centrifugation[4] was light, 1600 *g* for 6 min at 22 °C.

The PRP was transferred to an empty TOTM-PVC[1] bag through manual plasma extractor.[5] The PRP bag was subjected to a second spin[4] (3300 *g* for 8 min at 22 °C), to remove the excess of plasma. Manual extraction[5] was used and additive solutions[2,3] were added in the test groups in the proportion of 65% of PAS to 35% of residual plasma as recommended by the manufacturer's (32–47%) [12], with a final volume of about 65 mL (22 ± 2 mL of plasma and 42 ± 2 mL of PAS). The control group PCs (100% plasma) were prepared in the traditional way, by manual extraction[5] targeting the volume of approximately 65 mL. The compositions of the chosen PAS are shown in Additional file 1.

A sampling site coupler[6] was placed in all bags [13]. The calculated volume of PAS was added with the aid of sterile syringe and needle. The volume of the units was calculated in accordance with the specific gravity of resuspension solution (1.026 g/mL for plasma, 1.006 g/mL PAS) [3].

After this procedure, the bags were identified and kept at rest for an hour. Afterward, they were placed in a linear platelet shaker[7] located in a preservation chamber[8] in regulated temperature (22–24 °C) for 13 days.

Sampling

The PCs stored in each treatment were subjected to evaluation at established time points. The sampling was done by sampling site coupler[6] using syringe and sterile needles [14].

Analyzes were carried out on days 1, 5, 9 and 13. Platelet counts, MPV, PDW, swirling, pH, glucose, lactate, LDH, pO_2 and pCO_2, ATP, and flow cytometry to evaluate CD61, CD62P, Annexin V and JC-1 were assessed. Residual leukocyte count was performed on day 1 (24 h after collection). PCs samples were sent for microbiological culture on days 5 and 13.

Qualitative and morphological variables

Before the bag sampling, an evaluation of platelet swirling was performed. Swirling is a non-invasive method for assessing platelet viability; it is caused by light diffraction due to the alignment of normal discoid shaped platelets. In this evaluation zero indicates no swirling and 3 indicates great swirling [15]. Residual leukocyte count was performed using the Nageotte chamber.[9] The MPV, PDW and platelet counts were performed on an automated hematology counter.[10]

Surface markers and platelet function

Identification of platelet surface markers was performed through flow cytometry with monoclonal antibodies (MoAbs) CD61 FITC,[11] used for identification and quantification of platelet population; and p-selectin[12] (CD62P), for evaluation of platelet activation.

Gas analysis and metabolic variables

To assess platelet metabolism, the values of bicarbonate, glucose, pO_2 and pCO_2 were determined through a portable gas analyzer.[13] Also, in order to determine the ATP, samples were prepared in trichloroacetic acid solution, frozen at −80 °C [12], and measured by bioluminescence assay[14] aided by multi-mode microplate reader,[15]

according to the manufacturer's instructions. The determination of lactate was performed through dry chemistry.[16] The pH was measured by pH meter[17] according to the manufacturer's instructions. All samples were tested in duplicate.

Markers of apoptosis and mitochondrial potential

LDH was analyzed with dry chemistry[16]. The exposure of PS (phosphatidylserine) was assessed with annexin V marker[18] and the percentage of cells positive for the marker was quantified. Alterations in mitochondrial membrane potential ($\Delta\Psi m$) were determined with JC-1[19]evaluation. Relatives degrees of mitochondrial polarization were quantified by measuring the red-shifted (FL-2) JC-1 aggregates, which are favored under of high membrane potential.

All flow cytometric experiments were performed on a flow cytometer[20] where 30,000 events were acquired for each analysis[20]. Data were analyzed using FCS Express 5 software.[21]

Microbiological analysis

Inoculation was performed in BHI, with aerobic and anaerobic culture at 37 °C in samples from days 5 and 13 of assessment.

Statistical analysis

The quantitative data were expressed as the mean ± standard deviation. Two-way ANOVA was performed to analyse the effects of PAS and plasma. One-way ANOVA was performed to analyse the residual WBC count. When indicated, a post-hoc Tukey test or Duncan's multiple range test were performed. The analyses were performed through Graph Pad Prism 6.0.[22] The results with $p < 0.05$ were considered significant. Pearson correlation between variables was performed, considering significant values of $p < 0.05$.

Results

Composition of platelet concentrates

In vitro quality parameters were evaluated on day 1 (Table 1). All PCs had leukoreduction, average volume, pH, swirling and platelet concentration according to the quality control recommendations of regulatory authorities [16], and there was no significant difference between treatments. Three bags were excluded due to a contamination by *Staphylococcus spp.* negative coagulase on days 5 (one from each PAS) and 13 (Composol unit).

In vitro evaluation during the stock

Platelet count remained stable over storage time and there was no difference between plasma and PAS groups (Additional file 2). Regarding the swirling, a significant reduction in this parameter was observed over time ($p < 0.001$). The swirling was kept 2+ (suitable for use) until day 9 in both PAS, and until day 13 in the PC stored with SSP+ (Fig. 1).

The PDW and MPV morpho-structural rates showed differences between the days of evaluation. Both parameters had a significant increase in days 5, 9 and 13 compared to day 1 ($p < 0.001$) (Fig. 1), and there was a significant PDW increase in the control group on day 13 ($p < 0.001$) compared to the PAS groups.There was no significant difference in platelet count and labeling with CD61 along time or between groups. The percentage of CD61 positive cells remained above 85% (Additional file 2). The platelet activation evidenced by CD62P was higher in the control group compared to PAS ($p < 0.001$), with no difference among the PAS groups. There was an increased CD62P on day1 ($p = 0.014$) in all treatments, and on day 13 in the control group ($p < 0.001$).

On day 1, the control group showed higher pH values in comparison to the PAS groups (Fig. 1), however, the control group showed significant decrease along the storage time. Meanwhile in Composol and SSP+ there was an increase of pH during long-term storage. In Composol solution, pH was below the acceptable (< 6.2) on days 5, 9 and 13. The units held in SSP+ showed a more stable pH during the 13 days of storage (Fig. 1, Additional file 2).

A decrease in ATP concentration was observed in all groups, there was no difference between the treatments (Fig. 1). A decline of glucose consumption and an increased lactate production along the time was observed

Table 1 Residual leukocyte, platelet counts, swirling and pH on the experimental day 1

	Day 1		
	Plasma	SSP+	Composol
Volume (ml)	65.92 ± 1.977	64.05 ± 2.66	63.48 ± 3.11
Residual leukocytes (×10^6/unit)	0.0037 ± 0.0021	0.010 ± 0.009	0.005 ± 0.002
Platelet count (×10^{10}/unit)	7.197 ± 2.86	7.88 ± 2.13	7.64 ± 2.44
Swirling	3 ± 0	3 ± 0	2.86 ± 0.53
pH (22 °C)	7.162 ± 0.24 a	6.72 ± 0.20 b	6.44 ± 0.45 b

Different lowercase letters represent significantly different values ($p < 0.05$) between treatments. Results reported as mean ± standard deviation

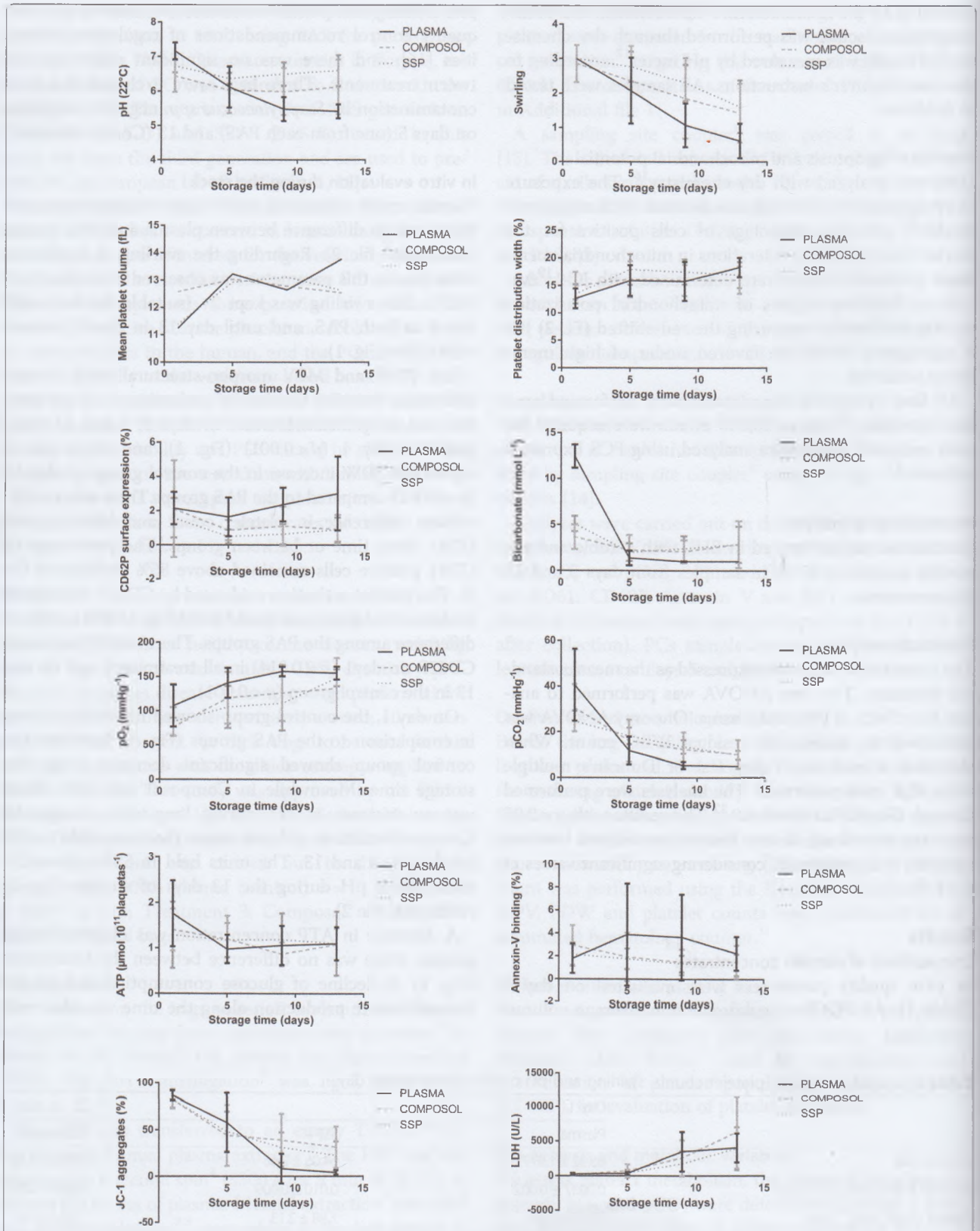

Fig. 1 Linear regression graphs of the in vitro parameters of plasma platelet concentrates and additive solution, stored for 13 days

($p < 0.001$), and the average of consumption/production was significantly higher in the control group compared to the PAS groups ($p < 0.001$) (Figs. 2 and 3, Additional files 3 and 4).

There was a decrease in bicarbonate concentration during the storage time ($p < 0.001$). The initial bicarbonate concentration was higher in the control group ($p < 0.001$), the decrease in concentration was consequently higher in this group compared to PAS (Fig. 1). A reduction in pCO_2 was observed during the storage time ($p < 0.001$) and the pO_2 behaved inversely over the storage time in all groups ($p = 0.001$)(Fig. 1).

There were no alterations in PS throughout the storage time, and there was a higher expression in the control group compared to SSP+ ($p = 0.007$) and Composol ($p = 0.050$) (Fig. 1). The LDH activity showed an increase over time in all groups (Fig. 1), being significant from the 9th day of storage, without differences between the groups ($p < 0.001$) (Fig. 1).

Regarding the $\Delta\Psi m$, there was a decrease in the percentage of JC-1 aggregates over the storage time ($p < 0.001$), leveling of between days 9 and 13. In the last 2 days of assessment, there was a significant lower percentage of JC-1 aggregates PC in the control group ($p < 0.001$) (Fig. 1).

Correlation study

The results of the correlations (Table 2) emphasize the findings described before, and are closely related to aerobic and anaerobic metabolism performed by platelets in the stock period. A decrease in glucose, pCO_2, HCO_3, ATP, pH and $\Delta\Psi m$ was observed along time; and the data show a positive correlation between these parameters. Also, a positive correlation interposes between the lactate production, cytoplasmic LDH release, increase of pO_2 and increased morpho-structural parameters - MPV and PDW.

Discussion

This is the first study to assess the feasibility to store canine platelets with PAS obtained by PRP method. The comparative study of PAS revealed that both solutions maintained the in vitro quality of canine platelets for up to 9 days after collection. Parameters such as swirling, pH and platelet counts were maintained for up to 13 days with the use of SSP+.

The results of metabolic evaluation, cell death, mitochondrial membrane polarization and activation support the in vitro stability evidenced in this study. The control group also remained stable until the fifth day, as expected, except for the average pH value (6.1 ± 0.59). The values recommended by regulatory authorities and studies in humans and canines were taken as a comparative basis. When canine PC parameters in plasma and PAS – produced by PRP method – were compared, similar data to those produced by buffy-coat method (BC) or apheresis in humans were observed. However this is the first study that report PC viability in PAS produced by PRP method (Table 3) [3, 14, 16–22].

All PAS require residual plasma to metabolism maintenance. The experiences of American and European studies indicate that when using PAS with less than 20% residual plasma through the BC method, there will be difficulty in obtaining blood component with the desired quality. Therefore, researchers suggest a residual plasma volume of 32–47% [8, 11, 12]. Our study showed adequate platelet parameters using 35% of residual plasma with manual extraction technique, suggesting that the technique is feasible and easy to execute.

Adequate platelet count is one of the most important pieces of information for clinicians, so it is a crucial point when producing a PC [23]. Studies that evaluated canine PC in 100% plasma observed a significant reduction of platelet count, it was associated with platelet activation and formation of microaggregates, fragmentation or loss of platelet integrity, and these may be the reasons of reduction of platelet count during the storage period [20, 21]. Our results are in agreement with previous studies evaluating human PC in SSP+ and Composol, where no variation in platelet count was observed [3, 15, 19]. Van der Meer (2012) stated difference in this parameter, the authors suggest that the low

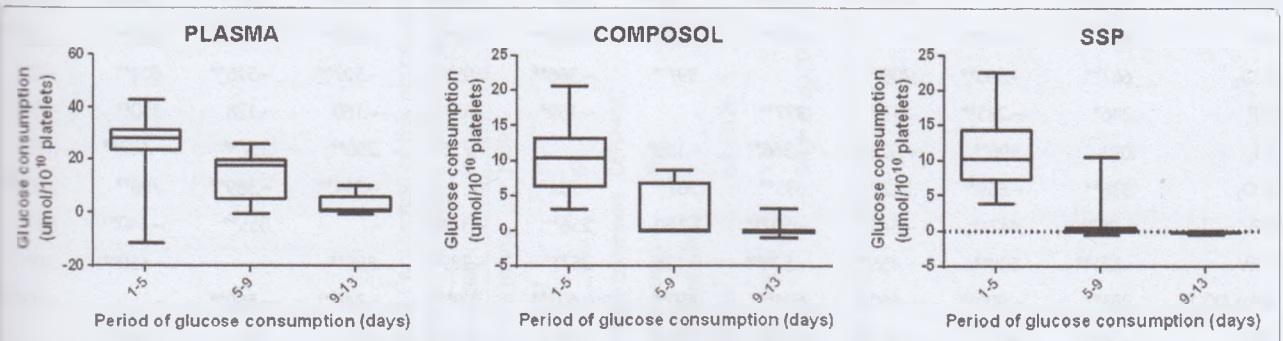

Fig. 2 Glucose consumption rates of platelet concentrates stored in plasma and additive solution for 13 days

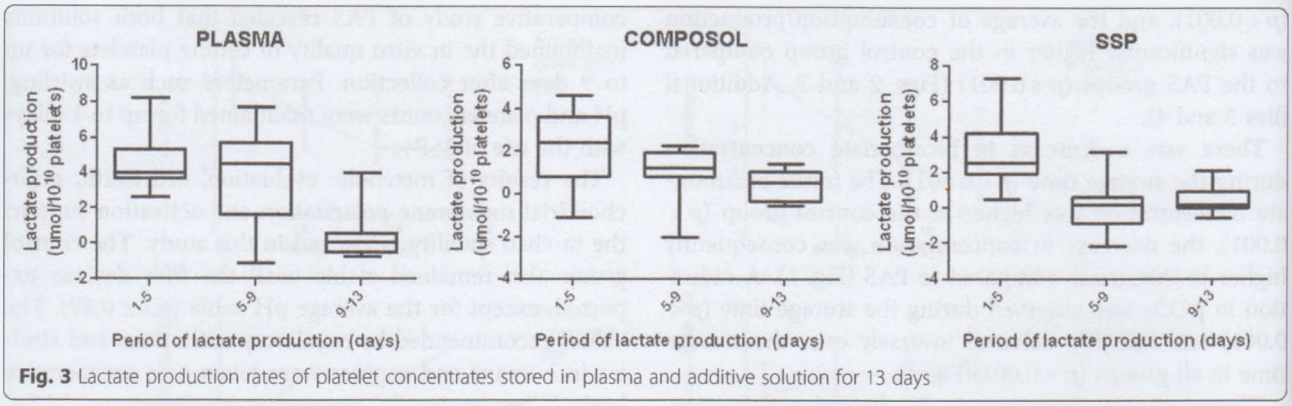

Fig. 3 Lactate production rates of platelet concentrates stored in plasma and additive solution for 13 days

viscosity of the PAS, makes it necessary to adapt and standardize the centrifugation protocols [24, 25].

Platelet swirling is used as a feasibility indicator to measure alterations in platelet morphology from discoid to spherical [12]. Corroborating the results of our experiment, other authors observed the maintenance of swirling with SSP+ and Composol for 7 days or longer (Table 3) [3, 11, 26].

In accordance with human PC data, alterations in MPV were not observed according to the used solution, this suggests that the change of format occurs regardless of the PAS used [7]. It is observed that the PDW in the control group has significant increase on the 13th day of storage which shows that the platelet fragmentation is more expressive in this group. These findings suggest that there is lysis and microparticle formation within the storage time [27, 28]. MPV showed a strong correlation with the pH decreasing over storage time, that indicate a loss of discoid to spherical shape, reflected in the MPV increase. Also we observed a strong correlation MPV and PDW with ΔΨm decreasing suggesting mitochondrial swelling and loose of potential (Fig. 1, Table 2) [29].

As a marker of platelet surface, the results of the use of CD61 are in agreement with other studies where the percentage of expression remained independent from the PAS used and the storage time [30, 31]. The CD62P is described with inconsistent results when correlating the increased expression with post-transfusion increment [29, 32]. In our experiment, the highest percentage of activation on day 1 was associated with the intense manipulation of the blood bag for its preparation, as described for human platelets. On day 13, CD62P expression was correlated with loss of platelet function [30].

When inferring the platelet metabolism, pH is one of the most used tools for quality control, turning into a mandatory assessment item at blood banks. When evaluating SSP+ and Composol with 35% of residual plasma in human PC, the pH maintenance was observed (>6.6) until the 8th day of storage (Table 3) [3, 30]. In agreement with previous studies, the pH decreased in all

Table 2 Pearson correlation between the metabolic characteristics of the stored plasma concentrates and the additive solution for 13 days

Correlations	Glucose	Lactate	pH	HCO_3	ATP	pO_2	pCO_2	MPV	PDW	ΔΨm (JC-1)	LDH
Glucose	–	−.220*	.468**	.667**	.246*	.093	.338**	−.283*	−.321**	.284**	−.226*
Lactate	−.220*	–	−.663**	−.590**	−.285**	.596**	−.659**	.477**	.500**	−.800**	.463**
pH	.468**	−.663**	–	.706**	.253**	−.525**	.542**	−.549**	−.455**	.660**	−.227**
HCO_3	.667**	−.590**	.706**	–	.397**	−.366**	.695**	−.507**	−.526**	.604**	−.073
ATP	.246*	−.285**	.253**	.397**	–	−.169*	.262**	−.160	−.128	.350**	−.099
pO_2	.093	.596**	−.525**	−.366**	−.169*	–	−.736**	.256**	.257**	−.602**	.179*
pCO_2	.338**	−.659**	.542**	.695**	.262**	−.736**	–	−.314**	−.389**	.708**	−.336**
MPV	−.283*	.447**	−.549**	−.507**	−.160	.256**	−.314**	–	.855**	−.440**	.017
PDW	−321**	.500**	−.455**	−.526**	−.128	.257**	−.389**	.855**	–	−.550**	.102
ΔΨm (JC-1)	.284**	−.800**	−.660**	.604**	.350**	−.602**	.708**	−.440**	−.550**	–	−.392**
LDH	−.226*	.463**	−.277**	−.073	−.099	.179*	−.336**	.017	.102	−.392**	–

*Correlation with significance level $p < 0.05$. **Correlation with significance level $p < 0.01$

Table 3 Comparison of this experiment with other studies data reporting in vitro parameters of CP in plasma, SSP + and Composol - using 35% of residual plasma for the PAS - in different processing methods, plastic bags material and storage times

Assessed data	Plasma					SSP+		Composol		
% residual plasma	100%	100%	100%	100%	100%	35%	35%	35%	35%	35%
PC method	PRP	PRP	PRP	PRP	PRP	PRP	BC	BC	BC	PRP
N	64	8*	22*	15*	13*	13*	35	23	10	14*
PC storage conteiners	DEHP-PVC	DEHP-PVC	TOTM-PVC	TOTM-PVC	TOTM-PVC	TOTM-PVC	Varied	Varied	Polyolefin	TOTM-PVC
Leukoreduction	Yes	No	No	No	No	No	Yes	Yes	Yes	No
Storage time (days)	5	7	7	9	5	9	8	8	9	9
pH (day 5)	6.7 ± 0.26	7.30 ± 0.3	6.7 ± 0.2	> 7.0	6.1 ± 0.59	6.36 ± 0.15	> 6.6	> 6.6	6.93 ± 0.12	6.04 ± 0.67
Swirling (day 5)	> 2	Uninformed	> 2	Uninformed	2.07 ± 0.95	2.76 ± 0.43	Appropriate		2.0 ± 0.7	2.35 ± 1
Reference	Singh et al., 2009 [22]	Hoareau et al., 2014 [46]	Costa et al., 2011 [47]	Sink, 2002 [48]	This study		van der Meer et al., 2010 [49]		Van der meer, 2001 [50]	This study

*Data from studies with dogs

canine PC units during storage, but it remained within acceptable limits (> 6.2) until day 13 just in the samples stored in SSP+. In other studies, this solution obtained pH above 7.0 on the 7th day of storage, using similar amounts of residual plasma (30%) [11, 31].

The choice of TOTM-PVC material as plastic for the bags of this study was based on results of previous studies which showed better pH stability in canine PC when compared to the use of DEHP-PVC [20]. Studies using DEHP-PVC bags consequently showed a lower pH during storage [33, 34]. Both materials are recommended for human PC storage in Brazilian blood banks [35].

The reduction of ATP levels observed in our study was correlated to a decrease of glucose and $\Delta\Psi m$ which results in the reduction of energy metabolism. When metabolically active, the platelet mitochondria produce ATP through anaerobic glycolysis which occurs in the cytosol as well as through the aerobic oxidation that occurs by the tricarboxylicacid cycle (TCA) [8, 36]. In reference to metabolism, it is highlighted that the PAS have low amount of glucose in order to reduce the rate of anaerobic glycolysis, which can be clearly evidenced by glucose consumption and lactate production rates. In comparison to the control group, the variation in lactate production in PAS occurs because of their emphasizing the aerobic oxidation. In our study, the results of the lactate and glucose rates refer to the same behavior of human PCs evaluated in plasma or PAS [37, 38]. Glucose consumption and lactate production are also stimulated by the presence of phosphate in the storage media. Therefore, the 100% plasma group showed significantly higher conversion rate compared to Composol or SSP+. Composol, which contains no phosphate in its formulation, showed the lowest conversion rates. The phosphate is present in SSP+, but the presence of potassium and magnesium counteracts its effect, and this provides low conversion rates similar to those observed with Composol [39]. There are no studies evaluating production and consumption rates in canine PC, but the studies that assessed glucose and lactate concentration observed metabolic behavior and values similar to those reported in humans [20, 21].

The bicarbonate concentration is influenced by the composition of PAS [39]. It is also observed that the bicarbonate concentration was more stable in SSP+; this pattern reflects the most stable pH values in this PC group. The same behavior was observed in another study comparing 100% plasma PC with 70% SSP+ for 14 days [38]. The presence of bicarbonate in the plasma has a buffering effect and in order to compensate the absence of this component, acetate was added to PAS. In TCA, acetate is metabolized to acetyl-CoA, consuming H+, and oxidized to CO_2 and H_2O, acting in a buffering mode against pH reduction caused by glycolysis [6, 36, 40].

A reduction in pCO_2 is observed along the storage time and pO_2 behaves inversely. Studies in human PC report an increase of pO_2 and a decrease in pCO_2 associated with the decline of oxidative metabolism [34]. Similar results to our study were obtained when comparing plasma to PAS PC in humans, and correlated pCO_2 values with the bicarbonate concentration of the medium [17, 30].

A previous study on PSL showed that the shelf-life and viability correlates with apoptosis mechanisms [41]. Therefore, the identification of intrinsic path markers (PS exposure, caspase marking) as well as extrinsic ones (quantification of cytochrome c, $\Delta\Psi m$) have been used. The PS exposure is considered to be an activation marker with pro-coagulant activity, and also an apoptosis indicator [1, 32]. In our study, no alterations during storage time were observed, however there is a greater expression in the control group on all the days of evaluation if compared to SSP+. In most studies, the expression percentage increases along time in 100% plasma PC and with the use of PAS, with a more significant expression in plasma [3, 38]. One experiment evaluated in vitro canine platelets for 8 days and observed an increase of PS only after the addition of apoptosis inducing agent in the environment. Previously to the effect of the inducing agent, the expression percentage was similar to that observed in canine PC from our study [42]. Our experiment partially reproduces the behavior observed in human PC, although there is no increase over time, we observed a greater expression in the 100% plasma group. This scenario may reflect a limitation of the technique used in dogs; or the fact that the exposure of PS is not a good marker of recent apoptosis in canine platelets.

LDH is a marker of senescence. As the metabolic potential of platelets is exhausted, platelet lysis occurs as well as there lease of cytosolic components including LDH [10, 32]. Studies in canine and human plasma PC and the use of PAS in human PCs demonstrate results that corroborate with those observed in our experiment [19, 20, 30]. This mechanism also reflects the positive results of Pearson correlation with lactate and negative results with pH and $\Delta\Psi m$ (Fig. 1, Table 2).

The assessment of mitochondrial potential is most commonly used when the goal is to assess interference of mitochondria in storage time or situations when the PC is not kept under constant stirring. There is few data concerning mitochondrial function and platelet viability with the use of PAS [29, 43]. The results presented here are in accordance with the evaluation of this parameter using SSP+ in human PC [38] and a single study in canine plasma PC (Lasta C.: Metabolism of canine platelets stored as platelet concentrate for 5 days, unpublished). Another group assessed Composol in human PC, observing mitochondrial viability from 12 days of storage, the results were consistent with our study; where there

is a drop of ΔΨm from the 9th day, which is more significant in the PC control group [10].

The bacterial contamination of 3 units was an obstacle. Coagulase negative *Staphylococcus* usually originates from the donor's skin and is often isolated in PC; and when it is transfused, it can cause fatal reactions associated with endotoxemia. Because of the intense manipulation, the care must be redoubled when the additive solution is added in the processing [5]. Two of the excluded units were from donors in which the owner did not allow trichotomy. The authors suggest that in these cases, the bags do not be used for CP production. However, it was not possible to determine if the contamination occurred at the time of the blood collection or during the sampling procedure of the blood bags. The contaminated samples were excluded from the study.

Correlations

The correlations enabled the identification of the main tendency among the variables used for platelet evaluation during storage. Platelet metabolism requires some direct correlations such as those between glucose and lactate, where the consumption of glucose directly results in lactate production, as well as between pH and pCO_2, where the pH was negatively correlated with pCO_2. Furthermore, the relationship between pO_2 and pH suggests that oxygen availability is strongly associated with maintenance of pH in an osmotic balanced environment. Some correlations are indirect, but they are intuitive between glucose/lactate and morpho-structural rates (i.e. MPV, PDW), apoptotic markers (i.e. LDH) and ΔΨm. These parameters represent the measure of platelet viability; low glucose or no glucose is usually the result of prolonged storage, which indirectly results in loss of viability. The main cause for the consumption of glucose, besides the regular metabolism, is the constant energy requirement to maintain the osmotic balance and the integrity of the platelet membrane, lost over time. This metabolic shift to aerobic glycolysis is probably responsible for the observed correlations between glucose and lactate (Warburg effect). Immune cells, as platelets, switch their energy production from oxidative phosphorylation to glycolysis upon cell activation. This metabolic programming has been attributed to facilitate cytoskeletal changes, increased ion signaling, enhanced phospholipid turnover, and greater macromolecule synthesis in a very short time during platelet activation [44]. There is a strong correlation among glucose, lactate, MPV, LDH and ΔΨm which indicates that the metabolic rate is related to degranulation, osmotic destabilization and/or swelling.

Conclusions

SSP+ and Composol seem to be an excellent alternative to replace plasma in the production of canine PC. Based on in vitro variables, canine PC storage in Composol is viable for 9 days, whereas the viability extends to 13 days with the use of SSP+. Prolonged storage, with in vitro quality maintenance, seems to be possible in veterinary medicine, with results similar to those seen in human platelets.

In vitro studies do not necessarily predict post-transfusion efficacy. Therefore, in vivo studies are needed to estimate the real contribution of PAS in preventing the PSL. This study provides a technical point of view needed for canine PC production with low plasma concentrations, and addition of PAS.

Endnotes

[1]CPD SAGM, Standard Handbags, JP Pharmaceuticals, BR
[2]PASIIIM, PAS-E, Macopharma, FR
[3]PAS-D, Fresenius Kabi, NLD
[4]Sorvall Legend RT centrifugal, Thermo Scientific, US
[5]ACS201, Terumo Medical do Brazil, BR
[6]Fenwal, Fresenius Kabi, US
[7]CDCI3, Indrel, BR
[8]AP48L, Presvac, AR
[9]LO-Laboroptik GmbH, DE
[10]Poch-100iV Diff, Roche Diagnostics Brazil, BR
[11]clone VI-PL2, BD Biosciences, US
[12]CD62P, AC1.2 clone, BD Biosciences, US
[13]CG8, ISTAT Abbott Point of Care, CA
[14]Lyte Luminescence ATP detection 1,010,224, Perkin Elmer, NL
[15]SpectramaxM5, Molecular Devices Inc., US
[16]Vitros 250 Chemistry System, Ortho Clinical Diagnostics, Johnson & Johnson, BR
[17]HI 9126, Hanna Instruments Inc., US
[18]ApoFlowEx FITC Kit, Exbio, CZ
[19]MitoScreen JC-1, BD Biosciences, US
[20]FACSCalibur™ and CellQuest™ Pro software, BD Biosciences, US
[21]De Novo, Software, US
[22]GraphPad Software, US.

Additional files

Additional file 1: Composition of the solutions used in the experiment.

Additional file 2: Platelet count, pH and CD61 percentage of positive cells mean ± standard deviation. Different lowercase letters represent significantly different values ($p < 0.05$) between treatments. Different symbols represent significantly different values ($p < 0.05$) between assessment days.

Additional file 3: Glucose consumption mean ± standard deviation of platelet concentrates stored in plasma and additive solution for 13 days. Different lowercase letters represent significantly different values ($p < 0.05$) between treatments. Different symbols represent significantly different values ($p < 0.05$) between assessment days.

Additional file 4: Lactate production mean ± standard deviation of platelet concentrates stored in plasma and additive solution for 13 days. Different lowercase letters represent significantly different values ($p < 0.05$) between treatments. Different symbols represent significantly different values ($p < 0.05$) between assessment days.

Abbreviations

ATP: Adenosine-5'-triphosphate; BC: Buffy-coat method; BHI: Brain-heart infusion medium; CBC: Complete blood count; CD61: Monoclonal antibodies (MoAbs) for GPIb expression; CD62P: p Selectin expression; CPD: Citrate phosphate dextrose; DEHP-PVC: Di(2-ethylhexyl)phthalate softener for polyvinyl chloride (PVC); FITC: Fluorescein isothiocyanate; JC-1: J-aggregate forming cation; LDH: Lactic acid dehydrogenase; MPV: Mean platelet volume; PAS: Platelet additive solutions; PC: Platelet concentrate; PDW: Platelet distribution width; PRP: Platelet rich plasma method; PS: Phosphatidylserine, annexin V marker; PSL: Platelet storage lesions; TCA: Tricarboxylic acid cycle; TOTM-PVC: Tri(2-Ethylhexyl) Trimellitate softener for polyvinyl chloride (PVC); $\Delta\Psi m$: Mitochondrial membrane potential

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Authors' contributions

NH, CSL planned and suited the project, performed the standardization of the tests, evaluation of donors and collection of blood bags, participated in experimental analysis, transcription and data analysis, were the major contributor in writing the manuscript. MLD, LAL standardized of biochemical and spin testing, evaluated of donors and collection of blood bags, substantial contributions to conception and design of the experiment, performed the evaluation of donors, interpretation of data. NAM, SRT, FBF standardized flow cytometry experiments, take part in experimental analysis, transcription, involved in drafting and critically revising the manuscript. DK, FHDG provide substantial contributions to conception and design of the experiment, interpretation of data, revising the manuscript. All authors were involved in drafting manuscript, ensure the accuracy and integrity of the data and give final approve to the version to be published.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Antimicrobial synergy between carprofen and doxycycline against methicillin-resistant *Staphylococcus pseudintermedius* ST71

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Abstract

Background: New therapeutic strategies are needed to face the rapid spread of multidrug-resistant staphylococci in veterinary medicine. The objective of this study was to identify synergies between antimicrobial and non-antimicrobial drugs commonly used in companion animals as a possible strategy to restore antimicrobial susceptibility in methicillin-resistant *Staphylococcus pseudintermedius* (MRSP).

Results: A total of 216 antimicrobial/non-antimicrobial drug combinations were screened by disk diffusion using a clinical MRSP sequence type (ST) 71 strain resistant to all six antimicrobials tested (ampicillin, ciprofloxacin, clindamycin, doxycycline, oxacillin and trimethoprim/sulfamethoxazole). The most promising drug combination (doxycycline-carprofen) was further assessed by checkerboard testing extended to four additional MRSP strains belonging to ST71 or ST68, and by growth inhibition experiments.
Seven non-antimicrobial drugs (bromhexine, acepromazine, amitriptyline, clomipramine, carprofen, fluoxetine and ketoconazole) displayed minimum inhibitory concentrations (MICs) ranging between 32 and >4096 mg/L, and enhanced antimicrobial activity of one or more antimicrobials. Secondary screening by checkerboard assay revealed a synergistic antimicrobial effect between carprofen and doxycycline, with the sum of the fractional inhibitory concentration indexes (ΣFICI) ranging between 0.3 and 0.5 depending on drug concentration. Checkerboard testing of multiple MRSP strains revealed a clear association between synergy and carriage of *tetK*, which is a typical feature of MRSP ST71. An increased growth inhibition was observed when MRSP ST71 cells in exponential phase were exposed to 0.5/32 mg/L of doxycycline/carprofen compared to individual drug exposure.

Conclusions: Carprofen restores in vitro susceptibility to doxycycline in *S. pseudintermedius* strains carrying *tetK* such as MRSP ST71. Further research is warranted to elucidate the molecular mechanism behind the identified synergy and its linkage to *tetK*.

Keywords: Veterinary antimicrobial therapy, Non-steroidal anti-inflammatory drugs, Multidrug resistance, Dogs

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Background

Occurrence of methicillin-resistant staphylococci in animals is a reason for concern in relation to both public and animal health [1]. In small animal veterinary medicine, infections caused by methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) pose a major therapeutic challenge since some MRSP strains, such as the European epidemic clone sequence type (ST) 71, are virtually resistant to all systemic antimicrobial products licensed for use in dogs [2]. As it is unlikely that new antimicrobial classes active against MRSP will enter the veterinary drug market in the near future, new therapeutic strategies are needed to exploit the current antimicrobial arsenal. Combination therapy is one of the possible strategies that can be used to manage severe MRSP infections that cannot be cured by topical antiseptic treatment. Some antimicrobial combinations such as amoxicillin clavulanate and potentiated sulphonamides are widely used in human and veterinary medicine. Research is warranted to identify new combinations of drugs acting on different targets concurrently. It has been hypothesized that combination antimicrobial therapy may prevent or delay development of resistance [3]. Promising results have been shown by combining antimicrobials with small non-antimicrobial helper molecules interfering with resistance [4].

Pharmaceutical preparations targeting eukaryotic cells and used for management of non-infectious diseases, hereafter defined as non-antimicrobial drugs, represent an unexplored source to potentiate existing antimicrobials, restore susceptibility against resistant strains or allow new uses and indications. Various non-antimicrobial drugs have shown in vitro antimicrobial activity [5] but their potential use in combination with existing antimicrobial drugs has never been tested systematically on veterinary pathogens. The objective of this study was to identify synergies between antimicrobial and non-antimicrobial drugs commonly used in small animal veterinary medicine as a possible strategy to restore antimicrobial susceptibility in MRSP. This objective was achieved by i) a double disk diffusion primary screening of six antimicrobial and 36 non-antimicrobial drugs, ii) minimum inhibitory concentration (MIC) testing of selected non-antimicrobials displaying antimicrobial activity and interaction with one or more antimicrobial disk in the primary screening, and iii) checkerboard secondary screening to assess synergy of the selected antimicrobial/non-antimicrobial combinations using a model strain of MRSP ST71 resistant to all antimicrobials tested. The most promising combination was further investigated by growth inhibition analysis and checkerboard testing of additional MRSP strains.

Methods

Selection of antimicrobials and non-antimicrobials

Six antimicrobials were selected to represent the five antimicrobial classes most commonly used in dogs and cats: β-lactams [ampicillin (AMP) and oxacillin (OXA)], fluoroquinolones [ciprofloxacin CIP)], lincosamides [clindamycin (CLI)], tetracyclines [doxycycline (DOX)] and potentiated sulfonamides [trimethoprim/sulfamethoxazole (SXT)] [6]. Although amoxicillin is the most frequently used penicillin in clinical practice, AMP was used as a surrogate as recommended by Clinical Laboratory Standard Institute (CLSI) [7]. Similarly, OXA was used for testing methicillin resistance according to CLSI guidelines [7]. Although CIP is not licensed for veterinary use, this fluoroquinolone was used instead of enrofloxacin, which largely metabolized to ciprofloxacin under in vivo conditions [8].

Thirty-six non-antimicrobials used in small animal practice were selected based on data on veterinary usage of drugs in Denmark (VetStat) [9], recommendations on frequency of usage by veterinary professionals at the local university hospital, and availability of the active compounds. Table 1 lists clinical use, solvent and supplier for each non-antimicrobial used in the study.

Bacteria strains and media

MRSP ST71 strain E104 resistant to all six antimicrobials tested was used for primary and secondary screening. Checkerboard testing was extended to four additional MRSP strains including ST71 (E032 and E095) and another widely distributed multidrug-resistant MRSP clone, ST68 (E122 and E135). All strains were grown on blood agar (Oxoid, United Kingdom) and incubated overnight at 37 °C prior to testing. All tests were performed using cation-adjusted Mueller-Hinton agar or broth (Sigma-Aldrich, Germany) using *S. aureus* ATCC 29213 as quality control strain.

Double disk diffusion test (primary screening)

The strain inoculum was prepared and plated according to CLSI guidelines for disk diffusion [7]. The following disk concentrations were used: AMP (25 μg), CIP (10 μg), CLI (10 μg), DOX (30 μg), OXA (5 μg) and SXT (1.25/25 μg). One antimicrobial disk was tested for each plate. The antimicrobial disk was placed at the centre of the plate and disks impregnated with 20 μL of non-antimicrobial solution at standard concentration (2 g/L) were applied at a 5 mm of distance from the antimicrobial disk. An antimicrobial disk not surrounded by non-antimicrobial disks was used as control on the same plate. Following overnight incubation at 37 °C, the plates were read to detect interactions between antimicrobial and non-antimicrobial disks. A clear extension of the edge of the inhibition zone of the antimicrobial disk in

Table 1 List of non-antimicrobial drugs selected for this study

Non-antimicrobial drug	Clinical use	Solvent	Supplier
Prednisolone sodium phosphate	Immunosuppressant	Water	Maymó
Cyclosporine		DMSO	Sigma-Aldrich
Dexamethasone sodium phosphate		Water	Alfasan
Praziquantel	Anthelmintic	DMSO	Haupt Pharma
Ondansetron[a]	Gastrointestinal problems	DMSO	Sigma-Aldrich
Omeprazole		DMSO	Sigma-Aldrich
Ranitidine[a]		Water	Sigma-Aldrich
Metoclopramide[a]		Water	Dechra
Salbutamol/Albuterol	Respiratory problems	Water	Sigma-Aldrich
Fluticasone[a]		DMSO	Sigma-Aldrich
Theophylline[a]		Water	Sigma-Aldrich
Sildenafil[a]		DMSO	Sigma-Aldrich
Bromhexine[a]		DMSO	Zoopan
Carprofen	Pain and inflammation	DMSO	Chanelle
Meloxicam		DMSO	Dopharma
Phenylbutazone[a]		DMSO	Alfasan
Paracetamol/Acetaminophen[a]		Water	SP Veterinaria
Estriol	Urinary problems	DMSO	Haupt Pharma
Medroxy-progesterone actetate	Hormonal problems	DMSO	Alfasan
Levothyroxine		DMSO	Dechra
Thiamazol/Methiamazol		Water	Dechra
Trilostane		DMSO	Dechra
Osaterone		DMSO	Virbac
Methylergometrine/Methylergonovine[a]		Water	Sigma-Aldrich
Levetiracetam[a]	Epilepsy	Water	No data
Pimobendan	Heart failure	DMSO	Dechra
Digoxin[a]		DMSO	Kela
Atenolol[a]		Water	Kela
Captopril[a]		Water	Novartis
Furosemide	Diuretics	DMSO	Alfasan
Spironolactone		DMSO	Haupt Pharma
Clomipramine	Psychological effects	Water	Haupt Pharma
Acepromaxine		Water	Alfasan
Amitriptyline[a]		Water	Haupt Pharma
Fluoxetine hydrochloride[a]		Water	Sigma-Aldrich
Ketoconazole[a]	Antifungals	DMSO	Sigma-Aldrich

[a]Only registered for human use in Denmark

proximity of the non-antimicrobial disk was interpreted as a positive result.

MIC determination by broth microdilution

The MICs of seven non-antimicrobials displaying interaction with antimicrobial disks in the primary screening were determined by broth microdilution [7]. The following stock concentrations were prepared: bromhexine [8192 mg/L, 75 % dimethyl sulfoxide (DMSO)]; clomipramine, acepromaxine and ketoconazole (1024 mg/L, 20 % DMSO); carprofen (1024 mg/L, 1.6 % DMSO); amitriptyline and fluoxetine (1024 mg/L). Serial two-fold dilutions were prepared in 96-well round-bottom microtiter plates (Thermo Scientific). The range of concentrations tested was determined individually for each compound and ranged between 0.5 and 4096 mg/L.

Checkerboard assay (secondary screening)

Two-dimensional checkerboard assays [10] were used to assess synergy for seven antimicrobial/non-antimicrobial combinations selected by the primary screening. Carprofen was additionally tested with tetracycline (TET) to check if the synergy effect was antimicrobial class-specific. Fractional Inhibitory Concentration Indexes (FICI) were calculated for each combination to determine whether the effect was truly synergistic (ΣFICI ≤ 0.5), no interaction (ΣFICI >0.5–4) or antagonistic (ΣFICI >4.0) depending on drug concentration [11]. The highest concentration of antimicrobials and non-antimicrobials used for the checkerboard assays was twice the MIC. The highest concentration possible was used if non-antimicrobials could not be dissolved at the desired concentration. Two-fold dilutions were prepared and inoculated with the test strain according to the CLSI guidelines for broth microdilution [7]. After overnight incubation at 37 °C, plates were shaken at 1200 rpm for 1 min in a Bioshake XP (Quantifoil Instruments GmbH). The optical density of growth cultures was measured at 600 nm (OD_{600}) using a Powerwave XS (BioTek) operated by software Gen5. Percentages of growth inhibition were calculated for each well using the following equation:

$$\%\ \text{inhibition} = 100 - \left(\frac{Mean\ OD\ of\ treated\ culture}{Mean\ OD\ of\ untreated\ culture}\right) \times 100$$

Growth inhibition assay

The inhibitory effect of carprofen/DOX was evaluated by exposing the model strain in exponential growth phase to the two drugs alone and in combination. Drug concentrations approximating the peak serum concentration (C_{max}) achieved in dogs by standard dosage in single drug therapy were used for this assay. According to the scientific literature, the C_{max} of DOX is 2.74–6.32 mg/L upon oral administration of 5–10 mg/Kg [12], whereas the C_{max} of carprofen is 32.6–38 mg/L upon treatment with 4.0 mg/Kg [13]. Taking into consideration DOX pharmacokinetic (PK) data in dogs and pharmacodynamic (PD) properties against *S. pseudintermedius*, [12] the strain was exposed to 0.5 mg/L DOX and 16, 32 or 64 mg/L carprofen. Briefly, overnight culture of the strain was diluted to 0.05 at an optical density of 600 nm (OD_{600}), grown up to OD_{600} 0.4 (10^8 cell forming units (CFU)/mL) and diluted again 1:1000 (10^5 CFU/mL). For the next 12 h, samples were collected every hour to perform standard bacterial counts. At OD_{600} 0.1 (10^7 CFU/mL, approx. after 3.5 h) aliquots of the culture were transferred into small flasks. Individual cultures were exposed to the selected concentrations of each drug alone or in combination and further incubated with untreated control. All cultures were setup in triplicates and incubated in water baths at 37 °C with shaking at 180 rpm. The percentage of growth inhibition at a specific time point was calculated using the following equation:

$$\%\ inhibition = 100 - \left(\frac{Mean\ CFU\ of\ treated\ culture}{Mean\ CFU\ of\ untreated\ culture}\right) \times 100.$$

Results

Seven of the 36 non-antimicrobial drugs tested in the primary screening were shown to enlarge the edge of the inhibition zone of at least one antimicrobial disk: acepromazine (CLI, OXA), amitriptyline (AMP, OXA), bromexine (OXA), clomipramine (OXA), carprofen (AMP, DOX), fluoxetine (CIP, SXT) and ketoconazole (OXA, SXT). Ketoconazole displayed the highest antibacterial activity (MIC = 32 mg/L), followed by acepromazine, clomipramine and fluoxetine (MIC = 64 mg/L), amitriptyline and carprofen (MIC = 256 mg/L) and bromhexine (MIC > 4096 mg/L).

These seven non-antimicrobials were further tested by checkerboard assays in combination with the antimicrobial displaying the largest inhibition zone in the double disk diffusion assay. The ΣFICI of bromhexine was not determined because the drug could not be dissolved at a sufficient concentration to determine the MIC (MIC > 4096 mg/L). Carprofen displayed synergistic antimicrobial activity with DOX, whereas the other five antimicrobial/non-antimicrobial combinations showed no interaction (ΣFICI = 1.01–1.35). Additional checkerboard assays were performed to determine at which drug concentrations carprofen displayed synergy with DOX or TET. Synergy was observed in presence of 64 mg/L of carprofen and 0.25–1 mg/L DOX (ΣFICI = 0.31–0.5). At lower carprofen concentration (32 mg/L), synergy was only displayed in presence of 1 mg/L of DOX (ΣFICI index = 0.38), whereas at higher concentration (128 mg/L) no effect was observed in presence of 0.125–1 mg/L of DOX (ΣFICI = 0.53–0.75). No effect was observed by increasing the concentration of DOX up to 2 mg/L with carprofen concentrations ranging from 16 to 64 mg/L (ΣFICI = 0.56–0.75). The synergy patterns of carprofen and TET were similar to those observed for DOX, even though they were less pronounced and required higher antibiotic concentrations (8–16 mg/L), resulting in a ΣFICI between 0.38 and 0.5 (Fig. 1). Checkerboard testing of four additional MRSP strains (two ST71 and two ST68) revealed synergy for the two ST71 strains when DOX and carprofen were combined at concentrations of 0.5/64, 1/64, 2/64 or 2/32 mg/L. On the contrary, no synergy was observed for the two ST68 strains (Fig. 1). At DOX/carprofen concentrations achievable in dogs by single drug

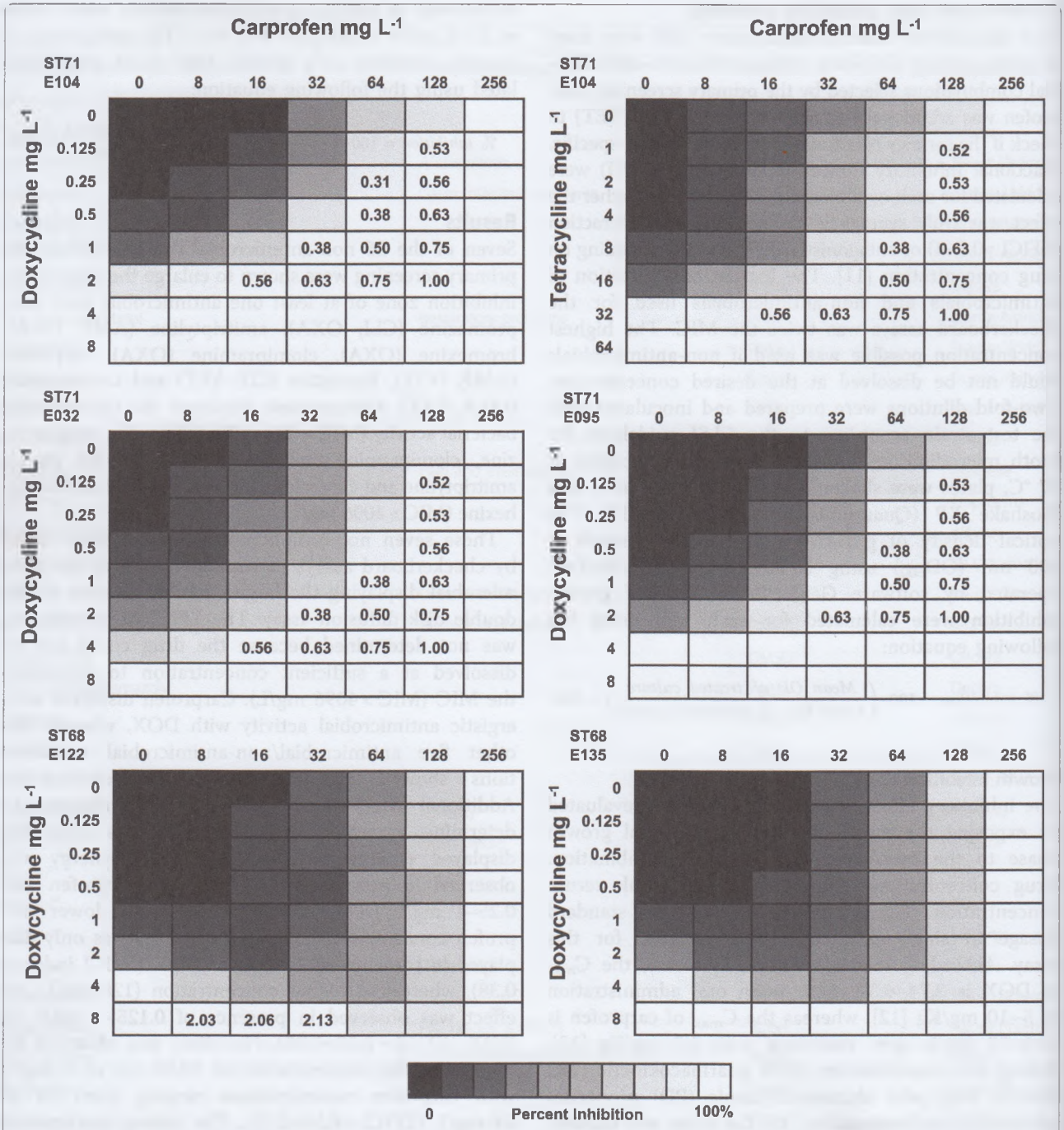

Fig. 1 Heat plots of the antibacterial effects of DOX or TET in combination with carprofen on three methicillin-resistant *Staphylococcus pseudintermedius* ST71 strains (E104, E032 and E095) and two ST68 strains (E122 and E135). The level of growth inhibition is expressed by colour intensity: the more intense the colour is, the less inhibited the strain was. The Fractional Inhibitory Concentration Index (FICI) was calculated for wells with no visible growth (white colour). Indexes of $\Sigma FICI \leq 0.5$ correspond to synergistic effect, $\Sigma FICI > 0.5$–4 no effect, $\Sigma FICI > 4$ antagonistic. The heat plots show the average of three biological replicates

therapy (0.5/32 mg/L), the percentage of growth inhibition measured by spectrophotometry was 54 % higher for the three ST71 than for the two ST68 strains.

Growth inhibition experiments were performed to assess the effect of carprofen and DOX, individually and in combination, during exponential growth of the model strain MRSP E104. Based on viable cell counts, the effect

of DOX and carprofen alone were significantly lower than for the combination of the two drugs (Fig. 2). After 4.5 h of exposure to 0.5 mg/L of DOX, the growth of strain was inhibited by 8.3 %; exposure to 16, 32 or 64 mg/L of carprofen alone resulted in 6.2, 26.6 and 40.9 % growth inhibition, respectively; exposure to 0.5 mg/L of DOX in combination with 16, 32 or 64 mg/L of carprofen inhibited growth by 45.7, 89.5 and 100 %, respectively, indicating a clear synergistic effect on growth inhibition by the drug combination.

Discussion

This study indicates that approximately 19 % of the 36 non-antimicrobial drugs tested were able to potentiate the antibacterial activity of one or more known antimicrobials against MRSP. All the seven non-antimicrobials that were found to have antimicrobial-potentiating activity (acepromazine, amitriptyline, bromexine, carprofen, clomipramine, fluoxetine and ketoconazole) have been previously reported to possess antibacterial activity [14–20]. Carprofen was shown to be particularly interesting to potentiate the antimicrobial activity of DOX as it displayed synergy at drug concentrations that may be achieved during therapy in dogs. Carprofen is a non-steroidal anti-inflammatory drug (NSAID) for veterinary treatment of inflammation and pain management. It has earlier been reported to have a clinical effect when used in combination with tilmicosin for antimicrobial therapy of bovine respiratory disease [21]. DOX is the most widely used tetracycline in small animal practice due to low systemic toxicity [22] and higher antimicrobial activity compared to TET [12]. However, due to widespread tetracycline resistance, DOX is presently regarded as a second choice antibiotic for most indications except upper respiratory tract infections [23].

The synergy between carprofen and DOX was studied in multiple MRSP strains, leading to the identification of

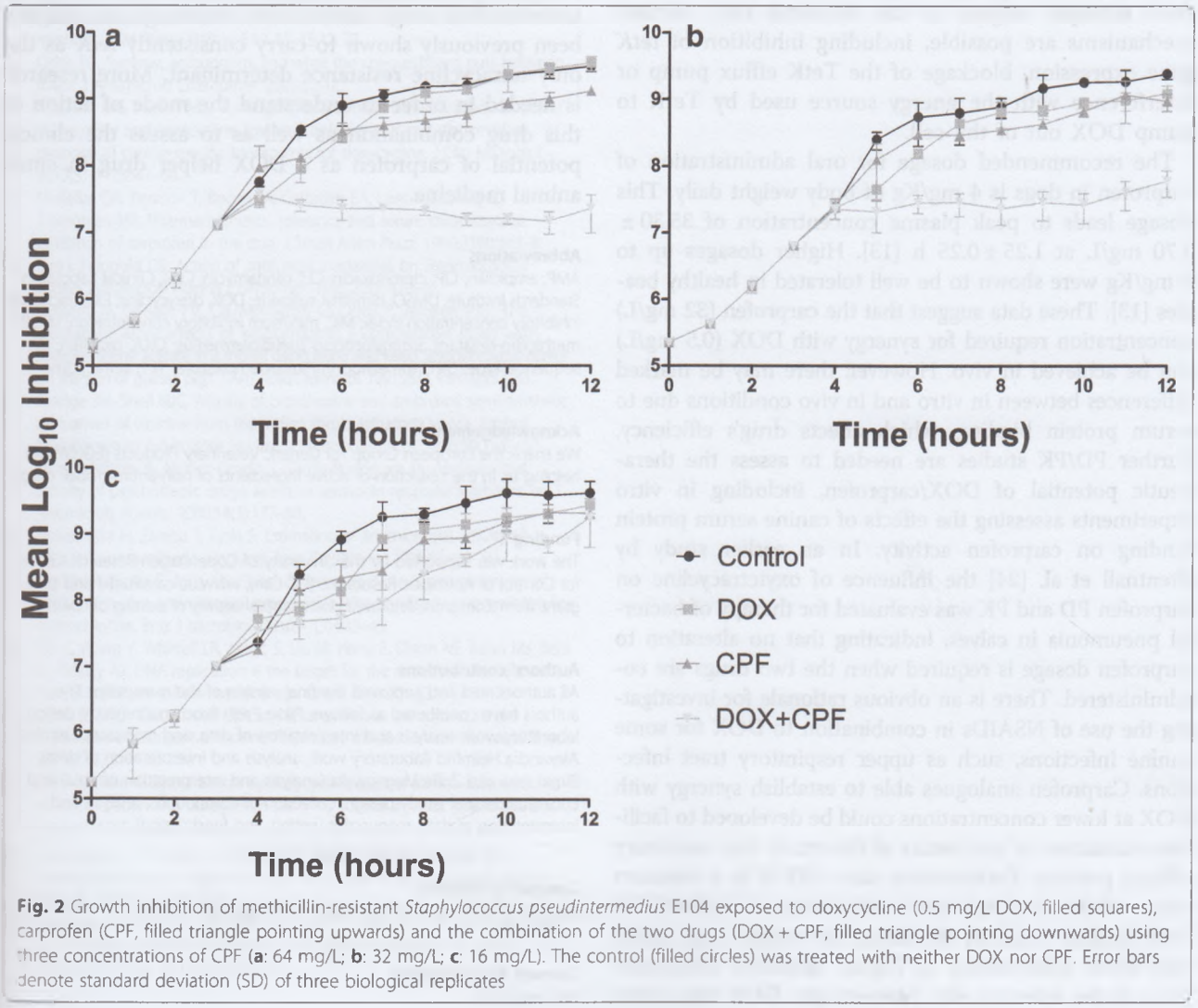

Fig. 2 Growth inhibition of methicillin-resistant *Staphylococcus pseudintermedius* E104 exposed to doxycycline (0.5 mg/L DOX, filled squares), carprofen (CPF, filled triangle pointing upwards) and the combination of the two drugs (DOX + CPF, filled triangle pointing downwards) using three concentrations of CPF (**a**: 64 mg/L; **b**: 32 mg/L; **c**: 16 mg/L). The control (filled circles) was treated with neither DOX nor CPF. Error bars denote standard deviation (SD) of three biological replicates

an association between the synergistic effect of this drug combination and strains belonging to the clonal lineage ST71, which harbours the efflux pump-mediated tetracycline resistance gene *tetK* [2]. In contrast, no synergy was observed for MRSP strains belonging to ST68, which are consistently associated with *tetM,* [2] an unrelated tetracycline resistance gene encoding ribosomal protection of the drug target. The association between *tetK* and DOX/carprofen synergy was further illustrated by the analysis of strain cultures exposed to concentrations of DOX/carprofen achievable in dogs by single therapy (0.5/32 mg/L), which showed a significantly higher growth inhibition in the three strains harbouring *tetK* compared to the two strains containing *tetM*. These results suggest that DOX/carprofen synergy only occurs in strains carrying *tetK*. The molecular mechanism behind the identified synergy and its linkage to *tetK* remains unknown. Such synergy mechanism is unlikely linked to the bactericidal effect of carprofen on DNA replication, suggesting that carprofen interacts with multiple targets in the bacterial cell. Various mechanisms are possible, including inhibition of *tetK* gene expression, blockage of the TetK efflux pump or interference with the energy source used by TetK to pump DOX out of the cell.

The recommended dosage for oral administration of carprofen in dogs is 4 mg/Kg of body weight daily. This dosage leads to peak plasma concentration of 35.30 ± 2.70 mg/L at 1.25 ± 0.25 h [13]. Higher dosages up to 9 mg/Kg were shown to be well tolerated in healthy beagles [13]. These data suggest that the carprofen (32 mg/L) concentration required for synergy with DOX (0.5 mg/L) can be achieved in vivo. However, there may be marked differences between in vitro and in vivo conditions due to serum protein binding, which affects drug's efficiency. Further PD/PK studies are needed to assess the therapeutic potential of DOX/carprofen, including in vitro experiments assessing the effects of canine serum protein binding on carprofen activity. In an earlier study by Brentnall et al. [24] the influence of oxytetracycline on carprofen PD and PK was evaluated for therapy of bacterial pneumonia in calves, indicating that no alteration to carprofen dosage is required when the two drugs are co-administered. There is an obvious rationale for investigating the use of NSAIDs in combination to DOX for some canine infections, such as upper respiratory tract infections. Carprofen analogues able to establish synergy with DOX at lower concentrations could be developed to facilitate translation of the results of this study into veterinary clinical practice. Furthermore, since MRSP is a common cause of skin and soft tissue infections, carprofen/DOX formulations could be developed for topical use, which may allow achievement of higher carprofen concentrations at the infection site. Interestingly, DOX has earlier been reported to have anti-inflammatory effects [25]. Thus, the combination of the two drugs might also have enhanced anti-inflammatory activity compared to single therapy.

The interactions between non-antimicrobial drugs and tetracyclines have occasionally been explored for potential clinical applications in human medicine. One example is the recent study by Ejim et al. [26] describing the synergistic effect of minocycline in combination with loperamide, a medication used for control of diarrhoea. Our study is the first attempt to investigate this alternative avenue for possible veterinary clinical applications.

Conclusion

The results show that carprofen is a potential antimicrobial helper drug to restore susceptibility to DOX in DOX-resistant MRSP strains carrying *tetK*. This finding is of clinical relevance since the epidemic multidrug-resistant clone MRSP ST71 is virtually resistant to all antimicrobial drugs licensed for veterinary use and has been previously shown to carry consistently *tetK* as the only tetracycline resistance determinant. More research is needed in order to understand the mode of action of this drug combination as well as to assess the clinical potential of carprofen as a DOX helper drug in small animal medicine.

Abbreviations

AMP, ampicillin; CIP, ciprofloxacin; CLI, clindamycin; CLSI, Clinical Laboratory Standards Institute; DMSO, dimethyl sulfoxide; DOX, doxycycline; FICI, fractional inhibitory concentration index; MIC, minimum inhibitory concentration; MRSP, methicillin-resistant *Staphylococcus pseudintermedius*; OXA, oxacillin; ST, sequence type; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline

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Authors' contributions

All authors read and approved the final version of the manuscript The authors have contributed as follows: Rikke Prejh Brochmann (study design, laboratory work, analysis and interpretation of data, and manuscript writing), Alexandra Helmfrid (laboratory work, analysis and interpretation of data), Bimal Jana and Zofia Magnowska (analysis and interpretation of data) and Luca Guardabassi (study design, collection of compounds, analysis and interpretation of data, manuscript writing, and fund raising).

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

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Effect of butorphanol, midazolam or ketamine on romifidine based sedation in horses during standing cheek tooth removal

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Abstract

Background: Standing surgery, especially dental procedures, are commonly performed in horses. This leads to an increasing demand for reliable sedation protocols. Therefore, it was the purpose of this study to investigate the influence of butorphanol, midazolam or ketamine on romifidine based sedation in horses during cheek tooth removal.

Methods: Forty horses presented for tooth extraction were divided in four groups using matched pair randomization. Group R was sedated with romifidine (bolus 0.03 mg/kg, followed by a constant rate infusion (CRI) 0.05 mg/kg/h) and group RB with romifidine (same dose) and butorphanol (0.02 mg/kg; CRI 0.04 mg/kg/h). Group RM received romifidine (same dose) and midazolam (0.02 mg/kg; CRI 0.06 mg/kg/h) whereas group RK was administered romifidine (same dose) and ketamine (0.5 mg/kg; CRI 1.2 mg/kg/h). If sedation was not adequate a top up bolus of romifidine (0.01 mg/kg) was administered. The quality of sedation and the conditions for tooth extraction, the level of ataxia, chewing, head and tongue movement were evaluated by using a scoring system. The investigator was blinded to the applied sedation protocol. Furthermore, serum cortisol concentrations before, during and after the procedure were analyzed to gain more information about the stress level of the horses.

Results: Horses in group RM showed significantly less chewing and tongue activity compared to horses sedated with romifidine alone or with butorphanol additionally, but also significantly higher levels of ataxia. The quality of sedation was significantly better if romifidine was administered in combination with ketamine compared to romifidine alone. Furthermore, horses of group RK needed less additional romifidine boli compared to all other groups. Blood cortisol concentrations during surgery in groups RB and RM remained unchanged. Horses of group R showed higher cortisol concentrations during sedation compared to horses of groups RB and RM.

Conclusion: Romifidine alone at an initial bolus dose of 0.03 mg/kg followed by a constant rate infusion of 0.05 mg/kg/h was insufficient to obtain an adequate level of sedation and led to increased stress levels, whereas the addition of butorphanol inhibited the stress response. The combination of romifidine with either midazolam or ketamine improved sedation quality and surgical conditions.

Keywords: Horse, Romifidine, Sedation, Midazolam, Ketamine, Cheek tooth removal

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Background

An increasing number of surgical procedures are conducted under standing sedation in horses [1]. Chemical restraint is usually obtained with a sedative often in combination with an opioid [2]. Bolus-administration followed by constant rate infusion (CRI) or repeated injections of boli are possible application methods. A CRI has the advantage of achieving a continuous plasma level and obtaining a constant depth of sedation [3], thereby reducing the adverse effects on the cardiovascular system [4].

Alpha-2 agonists are the most frequently utilized sedatives in equine medicine. Within this group romifidine achieves the longest duration of sedation [5]. Although the longer lasting sedative effect seems disadvantageous for an infusion, this compound produces the least ataxia of the currently available alpha-2 agonists [5]. This could be a major advantage for surgical procedures in the standing horse and outweighs the lower controllability of romifidine administered as CRI. The combination of opioids with sedatives induces a deeper and more reliable sedation than either drug alone [2]. The use of opioids alone is limited by the risk of inducing central stimulation and excitement, which might also contribute to "head bobbing" that is sometimes seen in horses after sedative combinations including opioids [6]. The agonistic activity of butorphanol at κ-opioid receptors as well as its competitive antagonist and agonist activity at μ- and δ-opioid receptors exerts the analgesic effect [7].

The benzodiazepine midazolam mediates muscle relaxant and anticonvulsant effects by its agonistic action on the inhibitory $GABA_A$-receptor in the brainstem, reticular formation and spinal cord [8]. Midazolam produces no sedative effect in adult horses [9]. Advantages of midazolam for dental procedures in horses have been described [10], but higher degrees of ataxia can occur [9, 10].

Ketamine is commonly used for dissociative anaesthesia in horses [11]. Recent studies showed beneficial analgesic potential of low dose ketamine in the standing horse [10, 12] without any sedative effect [13].

Cortisol concentrations can be used to quantify stress levels in humans and animals [14–16]. Physical restraint, manipulations in the mouth and pain represent potential stress-inducing factors for animals [15, 17]. All of these do occur during cheek tooth extractions. Therefore, plasma cortisol concentrations could be used as an indicator of insufficient sedation during surgery.

The aim of this study was to find a romifidine based sedation protocol for dental procedures, which produces reliable sedation allowing tooth extraction and minimizes stress reactions in horses.

Methods

Study design

Prospective clinical trial.

Animals

The study included 40 horses presented for cheek tooth extraction one to 2 days prior to surgery at the Clinic for Horses, University of Veterinary Medicine Hannover, Foundation. Horse owners gave informed consent for their animals' inclusion in the study and the study was approved by the institutional ethical committee of the University of Veterinary Medicine in Hannover, Germany. All horses were considered systemically healthy based on clinical examination. In the clinic they were fed hay and free access to water was provided.

Preparation

Three hours prior to sedation a 12-G intravenous catheter (INTRAFLON 2®)[1] was placed aseptically into the left or right jugular vein and meloxicam 0.6 mg/kg bwt (Metacam® 20 mg/ml)[2] was administered intravenously (i.v.). Neither food nor water was withheld prior to sedation. Horses were placed in stocks and left unhandled for 10 min for acclimatization. Self-adhesive electrode pads were attached to the thoracic wall of the horses to monitor the heart rate (HR) during the procedure via an electrocardiogram (Televet 100 - Telemetric ECG & Holter).[3] The respiratory rate (RR) was recorded based on visual observation. The relative head height was determined by measuring the distance from the rostral end of the nostrils to the ground.

Sedation protocols

Matched pair randomization was used to assign the horses to one of four different sedation protocol groups, thereby assuring proper homogeneity and comparability between groups. For example, if an 18-year-old Icelandic horse was assigned to group R, the next Icelandic horse or horse of the same age group was assigned to one of the three remaining groups. This procedure was continued until all four groups were assigned.

Assigned treatment groups were:

- Group R: Bolus romifidine (Sedivet® 10 mg/ml)[2] 0.03 mg/kg bwt i.v., followed by CRI romifidine 0.05 mg/kg bwt/h i.v.
- Group RB: Bolus romifidine 0.03 mg/kg bwt and butorphanol (Alvegesic® vet. 10 mg/ml)[4] 0.02 mg/kg bwt i.v., followed by CRI romifidine 0.05 mg/kg bwt/h and butorphanol 0.04 mg/kg bwt/h i.v.
- Group RM: Bolus romifidine 0.03 mg/kg bwt i.v. and midazolam (Midazolam B. Braun 5 mg/ml)[5] 0.02 mg/kg bwt i.v., followed by CRI romifidine 0.05 mg/kg bwt/h and midazolam 0.06 mg/kg bwt/h i.v.
- Group RK: Bolus romifidine 0.03 mg/kg bwt and ketamine (Narketan® 100 mg/ml)[6] 0.5 mg/kg bwt i.v., followed by CRI romifidine 0.05 mg/kg bwt/h and ketamine 1.2 mg/kg bwt/h i.v.

Drug dosages were based on a previous study from Hopster et al. [10]. The initial drug bolus was diluted in physiologic saline to a volume of 100 ml and administered over 10 min. The CRI medication was mixed with saline solution to a defined volume of 250 ml and was infused over a 1 h period. Continuous drug administration was ensured by drop counting by the investigator.

Local block

Ten minutes after the initial sedation bolus was administered, the maxillary or mandibular nerve was blocked with 0.4 mg/kg bwt lidocaine (Lidocainhydrochlorid 2%)[7] as described elsewhere [18]. Another 10 min later the mouth gag was introduced and the surgery was started (20 min after the beginning of sedation). The adequacy of the local block was tested by utilizing the gingival separator and the molar spreader. If horses reacted to this manipulation, the local block was assessed as inadequate and the nerve block was repeated. Oral extraction of the cheek teeth was performed in a standardized manner as described by Tremaine [19]. After completion of the extraction, the intravenous catheter was removed and horses were placed back in their stalls.

Measurements

All horses were assessed by one investigator who was blinded to the applied sedation protocol. Heart rate and RR were measured before the bolus application and in 10 min intervals thereafter. In addition the degree of ataxia, chewing and tongue activity as well as the head movement of the horses were graded by the investigator by means of a previously used scoring system (score 1–5; Table 1). If sedation was inadequate (scores for chewing/head movement/tongue activity ≥ 4), an additional bolus of romifidine (0.01 mg/kg bwt i.v.) was administered. After successful tooth extraction, the overall quality of sedation (overall behavior of the horses and degree of sedation) and quality of extraction (compliance of the horses to the surgical stimulus) was graded by the surgeon, who was blinded to the sedation protocol, using a visual analogue scale (VAS) from 1 to 10. One represented the best extraction or sedation quality and 10 described no signs of sedation and impossible cheek tooth extraction. All surgeries were performed by one of three surgeons, of whom one was a diplomate of the European Veterinary Dental College (EVDC) and two were final year residents of the European College of Veterinary Surgeons (ECVS). The relative head height was measured before and at 15 and 60 min after the initial sedation bolus. During relative head height measurements surgery was interrupted and horses were not handled.

Blood samples and cortisol analysis

Surgery was always performed in the morning between 9 a.m. and 12 p.m., to minimize the influence of the circadian cortisol rhythm [20]. Venous blood samples were collected from all horses three, two and 1 h as well as 15 min prior, and 15, 45, 60, 75, 90, 120, 150 and 180 min after bolus application (depending on the duration of the surgical procedure). During blood collection CRI was discontinued and 8 ml of blood were withdrawn from the intravenous catheter and discarded. Thereafter, another 8 ml of blood were obtained, transferred in serum tubes and the CRI was continued. At the end of the surgery, the CRI was stopped and two additional blood samples were taken after 20 and 120 min.

Each of the blood samples were incubated for 90 min at room temperature to allow complete clotting. Subsequently, the samples were centrifuged and the serum was stored at – 20°C prior to analysis.

Serum cortisol levels were assessed using a solid-phase, competitive, chemiluminescent enzyme immunoassay (Cortisol IMMULITE).[8] The measuring range extended from 1 to 50 µg/dl with an analytical sensitivity reaching 0.2 µg/dl.

Data analysis

Statistical analysis was performed with commercial software (SAS 9.2; SAS Inc., NC, USA). Gaussian distribution was tested by the Kolmogorov–Smirnov test and visual inspection of histograms. If data was not normally distributed non-parametric tests were used. Heart rate and RR were compared between groups and at different time points by one-way analysis of variance (ANOVA) with post-hoc Tukey test for multiple pair-wise comparisons. Differences in ataxia score were tested with

Table 1 Scoring system by Hopster et al. [10] to evaluate the degree of ataxia, chewing, head movement and tongue activity of horses

Score	Ataxia	Chewing/head movement/tongue activity
1	No signs of ataxia, even load on the limbs	No chewing/head movement/tongue activity
2	Mild ataxia, mild swaying, occasionally leaning against the stocks	Occasional chewing/head movement/tongue activity
3	Moderate ataxia, constant leaning against the stocks, buckling of the limbs	Continuously mild chewing/head movement/tongue activity
4	Severe ataxia constant leaning against the stocks, buckling of the limbs	Continuously severe chewing/head movement/tongue activity
5	Recumbency	No manipulation possible

permutation test and post-hoc Sidak-test for repeated measurements and evaluated for 100 min, when there were at least 6 horses in each group. The comparison of relative head height and sedation as well as extraction quality was determined by ANOVA. Head movement at different time points was analyzed by non-parametric ANOVA using the Kruskal-Wallis test followed by pair-wise comparison in between groups with Wilcoxon two sample test. Data of head movements were only analyzed as long as there were at least 5 horses in each sedation group left (this was the case until 60 min after beginning of surgery). Blood cortisol concentrations were compared by one way ANOVA and multiple pair-wise comparisons of the mean values. Significance level was set at $p \leq 0.05$ and data are presented as mean +/− standard deviation.

Results

The horses (29 Warmbloods, 7 ponies, 1 Thoroughbred and 3 Draft Horses) were between 3 and 27 years of age (mean 14 ± 6.4 years), weighing 313 up to 747 kg (mean 523 ± 108 kg). Detailed distribution of age, weight and gender between groups is listed in Table 2. There were no differences in distribution of gender and breed between groups. All 40 horses were healthy and their vital parameters were within normal limits.

Heart rate and RR significantly decreased after the sedation bolus in all horses and remained significantly lower compared to baseline values during the whole time of sedation (Table 3).

A significant ($p < 0.001$) decrease in relative head height was detected in all horses 15 and 60 min after bolus application compared to the pre-sedation head height. Further, relative head height was significantly lower at 60 min compared to 15 min after bolus application. No significant difference between groups was found. Relative head height in group R decreased after 15 min to 65.25 ± 15.7% of the baseline values and after 60 min to 45.38 ± 14.25% of the pre-sedation values. In group RB and RM the results were 60.62 ± 13.64%, 37.76 ± 16.28% and 70.65 ± 12.62%, 45.18 ± 10.37%, respectively. Horses sedated with romifidine and ketamine showed a decrease of the relative head height to 60.01 ± 13.56% after 15 min and to 44.5 ± 12.73% after 60 min.

Table 2 Distribution of age, weight and gender between groups

Sedation protocol	R	RB	RM	RK
Age	15 ± 6	13 ± 6	15 ± 6	14 ± 6
Weight	495 ± 74	496 ± 106	568 ± 135	534 ± 105
Gender	6 G, 4 M	4 G, 6 M	4 G, 6 M	4 G, 1 S, 5 M

Results for age and gender are presented as mean ± standard deviation
G gelding, *S* stallion, *M* mare, *Group R* Romifidine only, *group RB* Romifidine and Butorphanol, *group RM* Romifidine and Midazolam, *group RK* Romifidine and Ketamine

Table 3 Values for the heart and respiratory rate before and during sedation

Sedation protocol	R	RB	RM	RK
Heart rate baseline	53 ± 22	44 ± 8	47 ± 11	44 ± 7
Heart rate sedation	29 ± 4[a]	29 ± 2[a]	31 ± 3[a]	28 ± 3[a]
Respiratory rate baseline	22 ± 6	19 ± 3	22 ± 12	21 ± 6
Respiratory rate sedation	13 ± 3[a]	13 ± 3[a]	12 ± 3[a]	14 ± 5[a]

Results are listed as mean ± standard deviation
Group R Romifidine only, *group RB* Romifidine and Butorphanol, *group RM* Romifidine and Midazolam, *group RK* Romifidine and Ketamine
[a]= significant difference to baseline measurement

Baseline measurements for ataxia were not significantly different between horses. Horses of group RM developed a significantly higher degree of ataxia during sedation compared to baseline and compared to horses of group RB and RK (Fig. 1).

Horses of group RM showed significantly less chewing than horses of group R (time point 30) and horses of group RB (time points 20, 30 and 50). Head movements of horses were not significantly different between treatments. Tongue activity scores were significantly lower in horses of group RM compared to groups R and RB at time points 30 and 50 min.

Numbers of romifidine boli necessary to deepen sedation are listed in Table 4. Time to the first top-up bolus was not statistically different among groups.

The sedation quality was considered to be significantly better in group RK compared to group R (Table 4). Median values for extraction quality are also listed in Table 4. In two horses of group R tooth extraction was not possible, because repeated romifidine boli failed to prevent defensive movement and did not enable surgery. The results of the evaluations of these two horses were included until the CRI was stopped and surgery was continued under general anesthesia. No significant differences in extraction quality between groups were detected.

Cortisol analysis

Baseline measurements at 180, 120, 60 and 15 min before sedation revealed no statistically significant differences in serum cortisol concentrations between groups. Baseline concentrations were 49.34 ± 12.37 ng/ml, 55.95 ± 19.35 ng/ml, 51.9 ± 6.2 ng/ml and 56.27 ± 25.72 ng/ml in group R, group RB, group RM and group RK, respectively. In group R a significant increase above baseline was noticed 75 and 90 min after bolus application (Fig. 2). In group RK a significant increase in serum cortisol concentrations was detected 90 min after bolus administration compared to baseline measurements. No significant variations in cortisol concentrations were detected in groups RB and RM during sedation and tooth extraction.

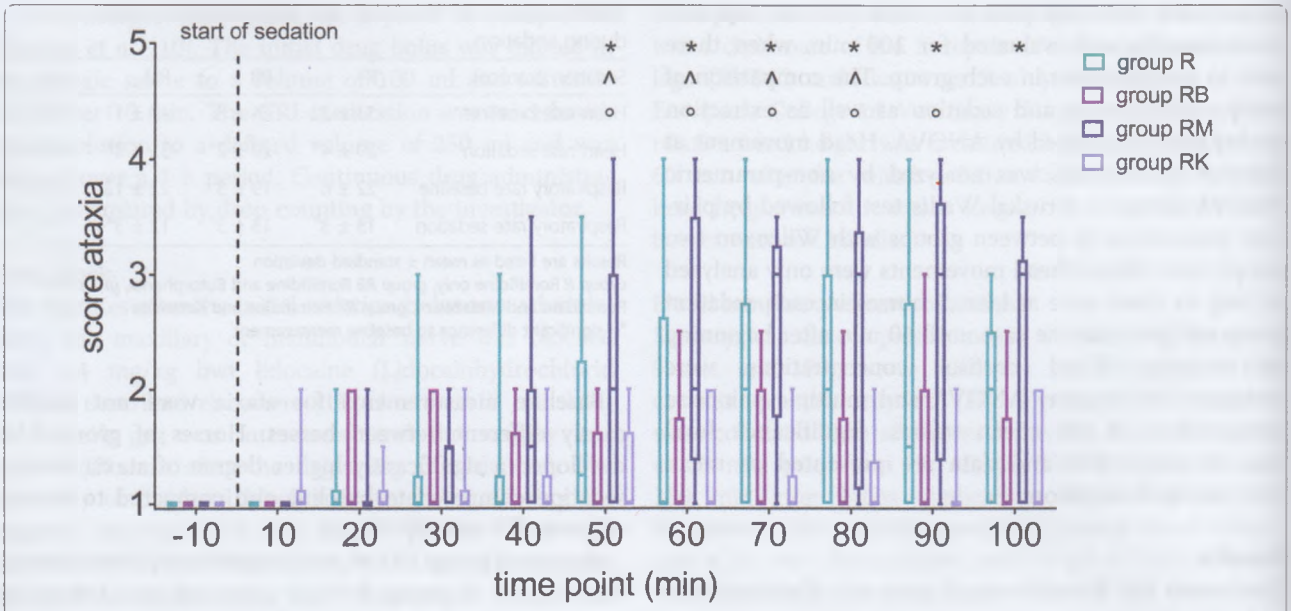

Fig. 1 Degree of ataxia in 40 horses being sedated with romifidine (group R), romifidine and butorphanol (group RB), romifidine and midazolam (group RM) or romifidine and ketamine (group RK). Score values for ataxia are presented as qq-plots at the different time points. A score value of 1 stands for no signs of ataxia, whereas 5 describes recumbency (Table 1). Boxes and whiskers demonstrate interquartile range and the minimal/maximal values. * = significant difference between group RM and baseline; ^ = significant difference between group RB and RM; ° = significant difference between group RK and RM

Twenty minutes after discontinuing drug infusion, serum cortisol values in group R, RM and RK increased significantly above baseline values. In groups RM and RK cortisol concentrations returned to baseline concentrations 120 min after surgery. Horses of group RB showed no significant changes in cortisol levels during the postoperative period. Overall, cortisol concentrations were not significantly different among groups.

Discussion

The four applied sedation protocols were able to produce chemical restraint for cheek tooth extraction in horses, except for two horses that were sedated with romifidine alone. However, in each group additional romifidine became necessary to perform dental surgery. For future clinical settings, an adjusted romifidine dosing regime is recommended.

In all horses the sedation resulted in a decrease in HR and RR, which is a well-known side effect of alpha-2-agonists [4, 21]. One horse in group R developed a HR below 20 beats per minute after receiving a total of six additional romifidine boli, which supports the fact that alpha-2 agonists mediate their cardiovascular depression in a dose-dependent manner [22]. There was no significant difference in HR between groups despite the trend in group R towards needing a higher total romifidine dose. This can be explained by the overall relatively low dose of romifidine that was administered.

Relative head height has been used as an indirect measurement for depth of sedation in horses [3].

Table 4 Sedation and extraction quality for 40 horses using a visual analogue scale (1–10)

Sedation protocol	R	RB	RM	RK
Sedation quality	5 (1–9)	5 (2–7)	4 (1–6)	3 (1–5)[a]
Extraction quality	5 (1–10)	4 (1–7)	3 (1–8)	3.5 (1–9)
Top-up boli of romifidine	3.5 ± 2.1	2.3 ± 2.4	2.8 ± 3.2	2.2 ± 2.1
Horses with top-up boli	9/10	7/10	9/10	7/10
Time to first top-up bolus (min)	7.8 ± 10.6	20.6 ± 19.1	22.8 ± 15.8	17.8 ± 10.7
Adjusted CRI (mg/kg/h)	0.071	0.064	0.067	0.063

Scores are given as median (min - max). One represents the best extraction or sedation quality and 10 describes no signs of sedation and impossible cheek tooth extraction. The mean and standard deviation of required top up boli of romifidine (0.01 mg/kg bwt i.v.) for each group is also listed as well as the number of horses that needed additional sedation. Additionally the time to the first bolus application and the adjusted constant rate infusion (CRI) are mentioned
Group R Romifidine only, *group RB* Romifidine and Butorphanol, *group RM* Romifidine and Midazolam, *group RK* Romifidine and Ketamine
[a]= significant difference to group R

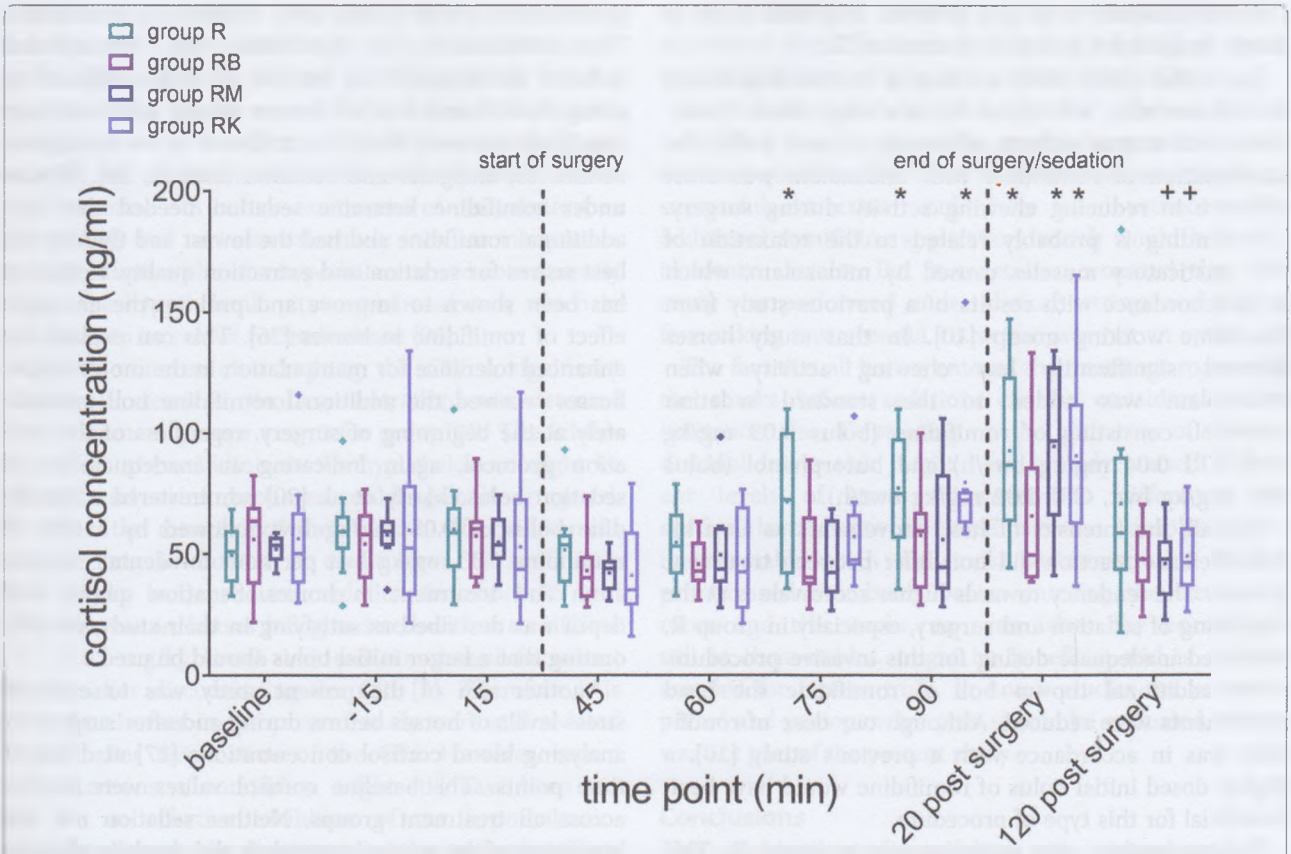

Fig. 2 Serum cortisol concentrations (ng/ml) of 40 horses before, during and after cheek tooth extraction. Cortisol values are presented as qq-plots at different time points. Boxes and whiskers demonstrate interquartile range and standard deviation. Means are presented as points; values exceeding 1.5 times the interquartile range are presented as rhombus'. Group R= Romifidine only; group RB= Romifidine and Butorphanol; group RM= Romifidine and Midazolam; group RK= Romifidine and Ketamine. * = significantly different to baseline values; + = significantly different to the previous time point

Reduction in relative head height was evident under all of the four different protocols without differences between groups. Horses receiving romifidine and butorphanol showed a tendency towards carrying their head lower compared to the other groups. This might be due to synergistic effects of butorphanol on sedation with romifidine [2, 3]. On the other hand, an increase in relative head height caused by the central excitement induced by midazolam [23] and ketamine [24] cannot be ruled out in groups RM and RK.

A side effect of alpha-2 agonists is dose-dependent ataxia [5, 21]. Romifidine produces less ataxia compared to other alpha-2 agonists [5, 21], representing a considerable advantage for standing procedures. Horses in group R, RB and RK showed a satisfying ability to stand stable in the stocks. In contrast, horses sedated with romifidine in combination with midazolam were significantly more ataxic than horses in group RB and RK. Three horses of group RM even displayed severe ataxia with a total score of 4. The severe ataxia started about 40 min after bolus administration and continued until the end of CRI. The main reason for this increased ataxia is the skeletal muscle relaxation induced by midazolam [25]. Hubbell et al. [9] demonstrated that intravenous midazolam doses exceeding 0.1 mg/kg bwt in conscious horses can result in severe ataxia and might even lead to recumbency. Even though the applied midazolam dose in the present study was low compared to most studies, the concomitant romifidine administration should be considered as a potential enhancing factor. Special care should be taken in case of using midazolam in combination with alpha-2 agonists that produce more muscle relaxation than romifidine. Ataxia scores in horses only sedated with romifidine showed trends towards being higher than in horses that received combinations of romifidine with butorphanol or ketamine. The good standing stability in group RB and RK, nearly indistinguishable from baseline values, was unexpected. The addition of butorphanol to alpha-2 agonists is described to lead to more instability in standing horses [2, 3, 6]. In the current study, no significant increase in ataxia in group RB compared to the other groups was found. Iburg [26] described severe ataxia in horses placed in their stalls after administration of romifidine (0.05 mg/kg bwt i.v.) and ketamine (0.06 mg/kg bwt i.v.), which disagrees with the current results. Whereas Peterbauer et al.

[12] and Lankveld et al. [13] detected only mild ataxia in horses in the first 5 min under ketamine CRI.

Successful cheek tooth extraction in standing horses is substantially influenced by chewing, head movement and tongue activity of horses. In our study, the combination of romifidine with midazolam was most effective in reducing chewing activity during surgery. This finding is probably related to the relaxation of the masticatory muscles caused by midazolam, which is in accordance with results of a previous study from the same working group [10]. In that study horses showed significantly less chewing activity when midazolam was added to the standard sedation protocol, consisting of romifidine (bolus 0.03 mg/kg bwt, CRI 0.04 mg/kg bwt/h) and butorphanol (bolus 0.01 mg/kg bwt, CRI 0.02 mg/kg bwt/h).

Overall, the intensity of head movements as an arbitrary defense reaction did not differ between treatment groups. The tendency towards higher score values at the beginning of sedation and surgery, especially in group R, indicated inadequate dosing for this invasive procedure. After additional top-up boli of romifidine the head movements were reduced. Although our dose of romifidine was in accordance with a previous study [10], a higher dosed initial bolus of romifidine would have been beneficial for this type of procedure.

Tongue activity was more intense in group R. This might be related to the site of action of alpha-2 agonists. Muscle relaxation as well as ataxia are mediated via inhibition of alpha-2 receptors in the spinal cord [27], whereas the tongue muscles are innervated by the hypoglossal nerve [28] and therefore are less affected by romifidine. The addition of butorphanol or ketamine did not significantly reduce tongue activity. In contrast, horses sedated with romifidine and midazolam showed significantly less tongue movements, which can be explained by the centrally acting midazolam mediating muscle relaxation via stimulation of $GABA_A$-receptors [8].

Horses in group R showed trends towards receiving the largest number of top-up boli of romifidine. This resulted in an adjusted romifidine CRI of 0.071 mg/kg bwt/h compared to 0.064 mg/kg bwt/h in group RB, 0.067 mg/kg bwt/h in group RM and 0.063 mg/kg bwt/h in group RK. However, the larger amount of romifidine failed to gain better scores for measured head parameters compared to the other sedation protocols. Horses under romifidine midazolam sedation required on average 20% less additional romifidine boli than horses of group R. Reduced chewing and tongue activity improved the surgical conditions for cheek tooth removal and permitted a lower depth of sedation in combination with excellent extraction quality. Sedation quality was scored as "good", but the lack of sedative and analgesic actions of midazolam and more pronounced ataxia might have influenced assessment. The combination of romifidine with butorphanol reduced the required top up boli by 34% compared to group R, and only 7 of 10 horses needed additional top ups. This can most likely be attributed to the synergistic actions on analgesia and sedation level [2, 29]. Horses under romifidine ketamine sedation needed the least additional romifidine and had the lowest and thereby the best scores for sedation and extraction quality. Ketamine has been shown to improve and prolong the analgesic effect of romifidine in horses [26]. This can explain the enhanced tolerance for manipulation in the mouth. Most horses received the additional romifidine boli immediately at the beginning of surgery, regardless of the sedation protocol, again indicating an inadequate initial sedation bolus. Marly et al. [30] administered a romifidine bolus of 0.08 mg/kg bwt followed by a CRI of romifidine 0.03 mg/kg bwt per hour for dental examination and treatment in horses. Sedation quality and depth was described as satisfying in their study, corroborating that a larger initial bolus should be used.

Another aim of the present study was to evaluate stress levels of horses before, during and after surgery by analyzing blood cortisol concentrations [17] at different time points. The baseline cortisol values were similar across all treatment groups. Neither sedation nor the beginning of the surgical procedure did result in changes in cortisol levels. The cortisol values increased in group R and RK 75 min and 90 min after the initial sedation bolus, respectively. This was surprising because tooth extraction was expected to be a strong stimulus to trigger the hypothalamic-pituitary-adrenal axis [15, 17]. However, one should take into account that alpha adrenoreceptors are widely spread within the *nucleus paraventricularis*, which is part of the hypothalamus [31]. Activation of presynaptic alpha-2 adrenoreceptors by alpha-2 agonists, such as romifidine, can result in inhibition of noradrenergic neurotransmitters leading to diminished cortisol concentrations as described in horses after application of clonidine or detomidine [32, 33]. The horses included in this study also received meloxicam pre-operatively, which may reduce the pain level in horses. In horses of group R, cortisol levels increased during tooth extraction reaching significant differences compared to baseline at the end of the procedure. We conclude that romifidine sedation alone failed to prevent the surgically induced stress response in horses, whereas sedation protocols RB and RM successfully suppressed this stress response. The reason for the lower cortisol levels in group RB might be the analgesic action of butorphanol and an increased depth of sedation. In addition, a direct inhibitory effect of opioids on the hypothalamus is described [34]. Such a direct inhibitory effect is also described for benzodiazepines

[35] and might explain the low cortisol levels during sedation with romifidine and midazolam. Horses in group RK showed elevated cortisol values during surgery. These results were unexpected, considering the beneficial analgesic effect of ketamine and the good sedation and extraction evaluations of this protocol. However, ketamine can induce excitement of central nervous system [24] and can also selectively increase vasopressin concentrations within the hypothalamus and consequently trigger the hypothalamic-pituitary-adrenal axis, which in turn leads to increased cortisol release [36].

Twenty minutes after ceasing the CRI a significant increase in serum cortisol concentrations was detected in all treatment groups except for group RB. The inhibitory effect of romifidine on the hypothalamus might be reduced and stress responses could again be triggered. However, the duration of the sedative effect of romifidine is long in horses. A single dose of romifidine (0.04 mg/kg bwt) produces sedation for about 75 min [37], whereas in horses being sedated with a romifidine CRI (0.03 mg/kg bwt/h i.v.) sedation lasted for about 60 min after discontinuing the CRI [3]. Therefore it is likely that horses in the present study were still sedated 20 min after termination of the CRI. However, serum concentrations of romifidine might still have been too low for an effective inhibition of the hypothalamic-pituitary-adrenal axis. Furthermore, the analgesic effects of alpha-2 agonists require a higher plasma level than is necessary for sedation [38]. Therefore, it is possible that the horses had greater pain as the plasma levels of romifidine dropped despite residual sedation. Horses receiving butorphanol did not show any increased cortisol levels within the postoperative observation period. Butorphanol enhances and prolongs the analgesic and sedative properties of romifidine [2]. The last blood sample was taken 2 h postoperatively. At this time point all cortisol values were back to baseline except in group R, where cortisol levels were still elevated. Duration and intensity of cortisol elevation depend on the strength of the stress factor [15]. Therefore, it can be concluded that cheek tooth extraction was more distressing for the horses sedated with romifidine alone compared to the horses receiving butorphanol, midazolam or ketamine additionally.

A limitation of the study was the variability in duration of the procedure and the drug infusion time which is inherent to clinical trials. In case of incomplete maxillary or mandibular nerve blocks, the nerve infiltration had to be repeated before surgery could be continued, thereby leading to delays. The variable duration of the surgical procedure as well as ineffective anaesthesia resulting in varying levels of pain might have influenced the cortisol concentrations. Duration of surgery ranged from 108 min in group R to only 71 min in group RK. For the intraoperative measurements, we included only the first 60 min of surgery, however for the postoperative measurements the differences in duration of surgery could have had an impact on pain and stress evaluations.

Another limitation was the potential influence of the romifidine to up boli on the results for extraction and sedation qualities. It is most likely that in some horses a successful extraction would have failed without using additional romifidine application, which would have led to clearer results. But it must be accounted that the study was performed under clinical settings and therefore risking unsuccessful extraction was not an option.

The fact that all horses entered the treatment room and the stocks before sedation, can be considered as a limitation also. Depending on the character of the individual horse this might have already caused different levels of stress. However, no differences in cortisol levels were observed between samples taken in the stable or in the stocks. Administration of the primary sedation bolus before entering the treatment room and the stocks, or even when the horses are still in their stables, might be beneficial and is recommended for further trials. In future studies the recovery period and postoperative pain should be evaluated, which was not part of the present study.

Conclusions

The combination of romifidine with midazolam facilitated oral manipulations in horses although higher levels of ataxia can be expected. An improvement of sedation quality was also obtained by adding ketamine instead of midazolam. Standing sedation for cheek tooth removal with romifidine alone led to increased stress levels, whereas the addition of butorphanol inhibited cortisol elevations during and after surgery.

Endnotes

[1]Vygon, Ecouen, France
[2]Boehringer Ingelheim GmbH, Ingelheim
[3]Rösch & Associates Information Engineering GmbH, Frankfurt am Main
[4]CP-Pharma GmbH, Burgdorf
[5]B. Braun Melsungen AG, Melsungen
[6]Vétoquinol GmbH, Ravensburg
[7]bela-pharm GmbH & Co. KG, Vechta
[8]Siemens Medical Solutions, Bad Nauheim

Abbreviations

ANOVA: Analysis of variance; CRI: Constant rate infusion; Group R: Horses sedated with romifidine alone; Group RB: Horses sedated with romifidine and butorphanol; Group RK: Horses sedated with romifidine and ketamine; Group RM: Horses sedated with romifidine and midazolam; HR: Heart rate; i.v.: Intravenous; RR: Respiratory rate; VAS: Visual analogue scale

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Authors' contributions
TMM, KH, ABZ, SK designed the study. TMM carried out the clinical examinations and measurements, drafted the manuscript and created the figs. KR performed the statistical analysis. KH, ABZ and SK critically revised the manuscript. All authors read and approved the final manuscript.

Ethics approval
The protocol was reviewed and approved by an internal, institutional committee (University of Veterinary Medicine, Hannover). The protocol was also approved by the "Animal Welfare Committee" of the University of Veterinary Medicine, Hannover University. In our case this committee not only decided that the study protocol met all necessary requirements but also concluded that the study design met all requirements of good and common clinical practice regardless of the assignment to a specific group in this study. Therefore, step three, approval by the state committee, the "Ethics Committee for Animal Experiments of Lower Saxony" Germany, was not necessary for this specific study.
Before conducting the sedation and dental procedure all owners were informed about this study and gave their consent.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Blood acid-base status in impala (*Aepyceros melampus*) immobilised and maintained under total intravenous anaesthesia using two different drug protocols

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Abstract

Background: In mammals, homeostasis and survival are dependent on effective trans-membrane movement of ions and enzyme function, which are labile to extreme acid-base changes, but operate efficiently within a narrow regulated pH range. Research in patients demonstrating a pH shifts outside the narrow regulated range decreased the cardiac output and systemic vascular resistance and altered the oxygen binding to haemoglobin. These cardiopulmonary observations may be applicable to the risks associated with anaesthesia and performance of wildlife ungulates on game farms. The aim of this study was to compare blood pH changes over time in impala immobilised and anaesthetised with two different drug protocols (P-TMP - immobilisation: thiafentanil-medetomidine; maintenance: propofol-ketamine-medetomidine; P-EME – immobilisation: etorphine-medetomidine; maintenance: etorphine-ketamine-medetomidine). Additionally, we discuss the resultant blood pH using both the Henderson-Hasselbalch and the Stewart approaches. Two data collection time points were defined, Time1 before maintenance of general anaesthesia and Time 2 at end of maintenance of general anaesthesia. We hypothesise that blood pH would not be different between drug protocols and would not change over time.

Results: Significant differences were detected over time but not between the two drug protocols. Overall, the blood pH decreased over time from 7.37 ± 0.04 to 7.31 ± 0.05 ($p = 0.001$). Overall, over time arterial partial pressure of carbon dioxide changed from 51.3 ± 7.5 mmHg to 72.6 ± 12.4 mmHg ($p < 0.001$); strong ion difference from 44.6 ± 2.4 mEq/L to 46.9 ± 3.1 mEq/L ($p < 0.001$); anion gap from 15.0 ± 3.1 mEq/L to 10.9 ± 2.2 mEq/L ($p < 0.001$); and total weak acids from 16.1 ± 1.2 mmol/L to 14.0 ± 1.1 mmol/L ($p < 0.001$). The bicarbonate changed from 29.6 ± 2.7 mEq/L to 36.0 ± 4.1 mEq/L ($p < 0.001$); and lactate changed from 2.9 ± 1.5 mEq/L to 0.3 ± 0.03 mEq/L ($p < 0.001$) over time.

Conclusions: The profound increase in the partial pressure of carbon dioxide that worsened during the total intravenous anaesthesia in both protocols initiated a substantial metabolic compensatory response to prevent severe acidaemia. This compensation resulted in a clinically acceptable mild acidaemic state, which worsened over time but not between the protocols, in healthy impala. However, these important compensatory mechanisms require normal physiological function and therefore when immobilising ill or anorexic wild ungulates their acid-base status should be carefully assessed.

Keywords: Blood pH, Impala, *Aepyceros melampus*, Immobilisation, General anaesthesia, Henderson-Hasselbalch, Stewart approach

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Background

Enzymes are important for metabolism and regulation of organ function and are labile to extreme acid-base changes, but operate efficiently within a narrow regulated pH range [1–3]. Research in patients demonstrating a pH shifts outside the narrow regulated range show decreased cardiac output and systemic vascular resistance and altered the oxygen binding to haemoglobin [1, 2, 4]. These cardiopulmonary observations may be applicable to the risks and success of anaesthesia and performance of wildlife ungulates on game farms.

Blood pH regulation is complex and involves various buffering systems and compensatory responses that keep the resultant pH within an optimal range for the species of animal [4–8]. Changes in pH are due to a change in the hydrogen ion (H^+) concentration [6, 7].

The traditional Henderson-Hasselbalch approach and the Stewart physicochemical quantitative approach are used to interpret blood pH. The Henderson-Hasselbalch approach relates the blood pH to the constituents of the bicarbonate (HCO_3^-) buffering system ($CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$) using the following equation [9, 10]:

$$pH = pKa \text{ of } H_2CO_3 + \log_{10}([HCO_3^-]/[H_2CO_3])$$

Which has been adapted for clinical application by the following equation [11, 12]:

$$pH = pK_1' + \log_{10}([HCO_3^-]/S.PCO_2)$$

where pK_1' is the equilibrium dissociation constant of carbonic acid = 6.105 at 37.0 °C (human); S is the solubility coefficient of carbon dioxide in plasma = 0.0307 [mmol/L]/mmHg.

The Stewart approach suggests that the HCO_3^- and H^+ represent the effect rather than the cause of acid-base derangements. Furthermore, the Stewart approach is based on the dissociation of water (H_2O) to produce H^+ or hydroxide ions (OH^-) to maintain electrical neutrality within a solution (like blood) where there are independent variables (arterial partial pressure of carbon dioxide [$PaCO_2$], strong ion difference [SID], anion gap [AG], total weak acids [Atot]) and dependent variables (H^+, OH^-, HCO_3^-, CO_3^{2-}, weak acids [HA] and ions [A^-]) which influence the neutrality [13]. Any change in the independent variable will effect a change in the dependent variables to maintain electrical neutrality within the solution. Stewart's theory has led to a revised version of the blood pH equation as follows [11, 12]:

$$pH = pK_1' + \log\{([SID^+] - Ka[Atot]/(Ka + 10^{-pH}))/S.PCO_2\}$$

where pK_1' is the equilibrium dissociation constant of carbonic acid; S is the solubility coefficient of carbon dioxide in plasma; Ka is the effective equilibrium dissociation constant of weak acids, the value is species dependent ($Ka = 0.8 \times 10^{-7}$ where pKa = 7.08; calves [11]). When using the Stewart approach the veterinarian must measure ($PaCO_2$ using a blood gas analyser) or calculate the independent variables to help interpret the resultant blood pH. Strong ion differences, anion gaps and total weak acid concentrations may be calculated using frequently published equations in the veterinary literature (Table 1).

Table 1 Calculations used to calculate variables of interest to explain the acid-base balance in healthy impala (*Aepyceros melampus*) undergoing immobilisation and general anaesthesia using two different drug protocols

Variable	Equation used in study	Equation references	Unit	Ruminant values	Value references
SIDa	= ([Na^+] + [K^+] + [Ca^{++}])-([Cl^-] + [Lactate])	[4, 5, 37]	mEq/L	Calf: 39.3 ± 4.5	[18]
				Calf: 40.0 ± 2.0	[16]
SIDe	= 2.46 × 10^{pH-8} × $PaCO_2$ + albumin (g/dL) × (0.123 × pH − 0.631) + phosphate (mEq/L) × (0.309 × pH − 0.469)	[4–6]	mEq/L	Calf: 34.8 ± 4.8	[18]
				Calf: 40.0 ± 2.0	[16]
SIG	= SIDa-SIDe	[5, 6]	mEq/L	Calf: 0.0 ± 3.0	[16]
AG	= ([Na^+] + [K^+])-([Cl^-] + [HCO_3^-])	[4, 8, 14, 18]	mEq/L	Goat: 20.02 ± 0.5	[3]
				Goat: 12.62 ± 1.7	[17]
				Goat: 20.0 ± 3	[19]
				Goat: 17.1 ± 3.9	[14]
				Calf: 20.29 ± 4.5	[18]
Atot	= 2.25 × albumin (g/dL) + 1.4 × globulin (g/dL) + 0.59 × Phosphate (mg/dL)	[4, 5]	mmol/L	Calf: 18.2 ± 2.6	[18]
				Calf: 19.2 ± 6.1	[16]

SIDa apparent strong ion difference, *SIDe* effective strong ion difference, *SIG* strong ion gap, *AG* anion gap, *Atot* total weak acids in plasma, Na^+ sodium ion, K^+ potassium ion, Ca^{++} calcium ion, Cl^- chloride ion, HCO_3^- bicarbonate ion, *g/dL* grams per decilitre, *mEq/L* milliequivilent per litre, *mg/dL* milligrams per decilitre, *mmol/L* millimoles per litre

There is a growing body of literature that provide reference ranges for the independent variables in domesticated production ungulates, in healthy [14] and diseased states [15–21]. However, there is a paucity of information regarding ranges of these variables in wildlife ungulates. Furthermore, the effect of various immobilisation and total intravenous anaesthesia protocols on blood pH balance have undoubtedly not been explored. Field ready drug protocols to maintain surgical anaesthesia in wild ungulates is becoming increasingly important, due to the increased demand of completing invasive surgical procedures such as bone fracture repair [22, 23]. The drug protocol should be made up of commonly available drugs and be easy to administer. Furthermore, the combination should maintain the animal's organ physiology within clinically acceptable ranges to minimise compromising vital organ function [4, 5, 23].

The aims of this study were to measure and report the blood pH change over time in healthy adult female impala undergoing immobilisation and general anaesthesia using two different drug protocols. We hypothesise that blood pH would not be different between drug protocols and would not change over time. In addition, we aim to discuss the measured blood pH by describing the change in variables described by the Henderson-Hasselbalch and Stewart approaches of interpreting blood pH.

Methods

This study was a part of a larger series of studies exploring the feasibility and cardiorespiratory effects of two different immobilisation and total intravenous anaesthetic protocols (drug protocols) administered for 120 min. All studies were approved by the animal ethics and research committees of the University of Pretoria prior to data collection (V099–13 & V012–16). The feasibility and cardiorespiratory outcomes of the two drug protocols are reported elsewhere and their findings are independent of those reported here [22, 23]. The present study reports on the acid-base status of the impala undergoing the two drug protocols.

Ten adult non-pregnant female impala aged between 12 and 36 months old were enrolled in this prospective cross-over study. The impala were captured from a nearby game farm and transported to the Faculty 6 weeks prior to the drug trials. They were housed in a purpose built 2.7 m high walled outdoor enclosure (boma) for the duration of the study. The boma was divided, by an internal wall with swing gates at either end, into a small area used for daily feeding and a larger home area. A 6 week pre-trial period was used to allow the impala to familiarise themselves with the boma and daily husbandry routine [24]. The impala received hay (*Erogrostis curvula*), lucerne (*Medecargo sative*) and water ad libitum; commercially available antelope pellets (Alzu antelope pellets; Alzu; South Africa; approximately 100 g/animal/day) were supplemented based on observed body condition.

All impala received two drug protocols (P-TMP & P-EME) on two occasions separated by 4 weeks:

- P-TMP – Immobilisation: thiafentanil (0.05 mg/kg; Thianil 1%; Wildlife Pharmaceuticals; South Africa) and medetomidine (0.055 mg/kg; Medetomidine 1%; Kyron Prescriptions; South Africa); Maintenance: propofol (12 mg/kg/h; Propoven 1%; Intramed, South Africa), ketamine (1.5 mg/kg/h; Ketamine Fresenius 10%; Intramed) and medetomidine (0.005 mg/kg/h; Domitor 0.1%; Zoetis; South Africa) ([22]).
- P-EME – Immobilisation: etorphine (0.05 mg/kg; Captivon 0.98%; Wildlife Pharmaceuticals) and medetomidine (0.055 mg/kg); Maintenance: etorphine (0.04 mg/kg/h), ketamine (1.5 mg/kg/h) and medetomidine (0.005 mg/kg/h) ([23]).

The impala were immobilised in the same order, on the same day of the week (two impala per day), at approximately the same time of the day, as randomised in the first week of data collection.

All impala were enclosed in the smaller feeding partition of the boma prior to darting. The impala were remotely injected using a filled dart (3 mL air pressurised dart; Dan-Inject; South Africa) containing the immobilisation combination, projected into the muscles of the pelvic girdle via a carbon dioxide powered rifle (set to 5 bar pressure, 12–15 m darting distance; Dan-Inject; Model JM). Once the dart was placed and fully discharged, a stopwatch was started to record the times to sampling. When the impala was immobilised into a recumbent position without attempts to stand the remaining impala were released into the larger home area of the boma and the immobilised impala was approached. An initial field clinical examination was completed and a cannula was aseptically placed into one of the cephalic veins prior to vehicle transport to the procedure room approximately 650 m away. Once in the procedure room, the impala was instrumented with a number of monitoring devices to measure cardiorespiratory and temperature parameters throughout the 120 min total intravenous anaesthesia ([22, 23]). Simultaneously, while placing the monitoring devices, an auricular artery was aseptically cannulated for serial arterial blood sampling and direct arterial blood pressure monitoring. The impala were left to breathe spontaneously throughout the study. If apnoea (no attempt to breathe over a 60 s period) was detected at any time during the procedures, then butorphanol (1:1 potent opioid dose) was administered intravenously [22, 23]. All impala tracheas were intubated (size 8.0 polyvinyl chloride cuffed endotracheal tube) and received oxygen insufflation

(fixed rate of 2 L/min) via a nasogastric feeding tube (8 French Gauge; Avacare feeding tube; Sunray Medical; China) placed approximately to the level of the fourth intercostal space. Physiological saline (Sodium Chloride Fresenius 0.9%; Intramed; South Africa) was administered at a fixed maintenance rate of 5 mL/kg/h for the entire 120 min anaesthesia period.

Data collection of importance to the present study consisted of venous (lateral saphenous; needle and syringe technique; stored in serum tube) and arterial (aspirated from the auricular artery cannula using a pre-heparinised syringe and needle) blood sampling at two distinct time points. Time 1 was immediately prior to the start of the total intravenous anaesthesia infusion and oxygen supplementation, and Time 2 was 1 minute prior to cessation of total intravenous anaesthesia infusion, and before transporting the impala back to the boma for recovery. The times to sampling (from dart placement until sampling) for the two distinct times were recorded.

The venous sample was allowed to clot prior to centrifugation to separate the serum from the cellular components. The serum was carefully pipetted and stored in cryovials in a – 80 °C freezer until analysis. Serum phosphorus, albumin and globulin from the venous sample was analysed using a calibrated bench top serum analyser (Cobas, Integra 400 Plus; Roche Products (Pty) Ltd.; South Africa).

The arterial blood sample was collected and analysed immediately using a calibrated patient side blood gas analyser (EPOC Reader Blood Analysis Analyzer and EPOC BGEM smart cards; Epocal; USA). The blood gas analyser measured the following variables of interest: pH, $PaCO_2$, sodium, potassium, calcium, chloride and lactate, haematocrit and haemoglobin concentration. The base excess (BE) and bicarbonate (HCO_3) was calculated based on the analyser's internal algorithm setting for "other" species. All results were interpreted at a fixed body temperature of 37 °C (alpha-stat analysis). Rectal temperature (Physitemp Model BAT-12; Physitemp Instruments; USA) was continuously monitored and recorded at the time of blood sampling.

The impala were recaptured and transported back to their source on completion of the series of studies.

Data analysis

Data were assessed for normality by plotting histograms, calculating descriptive statistics and performing the Anderson-Darling test for normality. Variables of interest (electrolytes, arterial carbon dioxide tension, base excess, bicarbonate, lactate, strong ion differences, anion gaps, total weak acids, proteins, haematocrit, haemoglobin concentration and temperature) were compared between protocols and time (both fixed effects) where impala were modelled as a random effect using a general linear mixed model analysis. Independent variables that cause the change in pH over time (partial pressure of carbon dioxide, apparent strong ion difference, anion gap and total weak acids) are presented graphically using box plots and whiskers [13]. Correlation between blood pH and variables of interest (bicarbonate ion, partial pressure of carbon dioxide, apparent strong ion difference, anion gap and total weak acids) were assessed using Persons correlation. The times to sampling for the first and second sampling points were compared between protocols using the two-sample t-test. Results reported as mean ± standard deviation (SD). Overall values were reported as mean ± standard deviation of the combined data from both protocols at the two time points. Data were analysed using commercially available statistical software (MiniTab 17.1.0; MiniTab Incorporated; USA) and results interpreted at the 5% level of significance. The main null hypothesis tested was that there would be no difference in blood pH between protocols and over time within a protocol.

Results

The impala were weighed and the drug doses used for the immobilisation were recalculated on a per kilogram bases. In P-TMP, thiafentanil and medetomidine were dosed at 0.052 ± 0.007 and 0.057 ± 0.006 mg/kg, respectively. In P-EME, etorphine and medetomidine were dosed at 0.050 ± 0.012 and 0.054 ± 0.013 mg/kg, respectively. Both drug protocols immobilised the impala adequately. Within the first 15 min of recumbency butorphanol boluses were administered to eight impala in P-TMP and to three impala in P-EME that developed apnoea. Repeated butorphanol boluses were necessary in most impala receiving P-TMP. All impala were breathing regularly and spontaneously prior to Time 1 and no more butorphanol boluses were required.

One impala receiving P-EME sustained an inoperable comminuted fracture to a femur due to a darting injury and was humanly euthanised. Data collected from this impala were excluded from analysis.

The blood pH significantly decreased over time within both drug protocols (Table 2; $p = 0.001$), however, there was no significant difference between the two protocols at both the time points ($p = 0.974$; interaction: protocol x time). Overall, the pH changed from 7.37 ± 0.04 to 7.31 ± 0.05 at Time 1 to Time 2, respectively.

According to the Stewart approach, evaluation of the independent variables responsible for shifts of the hydrogen ion concentration, and thus blood pH, demonstrated statistically significant shifts over time that were of clinical interest (Fig. 1 and Table 2). The $PaCO_2$ ($p < 0.001$)

Table 2 Measured and calculated values obtained from healthy impala (*Aepyceros melampus*) undergoing immobilisation and general anaesthesia using two different drug protocols

Variable	Unit	Time 1				Time 2				*P* value
		P-TMP		P-EME		P-TMP		P-EME		
		Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	
Times to sampling (from dart placement until sampling)										
Times to sampling	min	16.8	±7.0	19.2	±5.6	150.4	±5.7	151.8	±6.3	
T-test *P* value		*P* = 0.442				*P* = 0.613				
Basic clinical parameters at time of sampling										
Heart rate	Beats/min	122	±40	79	±37	57	±9	59	±11	<0.001
Resp rate	Breaths/min	9	±5	9	±2	10	±2	10	±2	0.316
MAP	mmHg	126	±14	117	±18	102	±12	90	±19	0.001
Temperature	°C	38.9	±0.4	39.3	±0.2	37.0	±0.2	37.0	±0.2	<0.001
Arterial blood acid base analysis										
pH	N/A	7.36	±0.04	7.38	±0.04	7.31	±0.02	7.30	±0.05	0.001
HCO_3^-	mEq/L	29.0	±2.7	30.1	±2.9	36.0	±5.9	35.9	±1.5	<0.001
	mmol/L	29.0	±2.7	30.1	±2.9	36.0	±5.9	35.9	±1.5	
BE	mEq/L	3.6	±2.4	4.9	±3.1	9.8	±6.1	9.5	±1.5	<0.001
	mmol/L	3.6	±2.4	4.9	±3.1	9.8	±6.1	9.5	±1.5	
Lactate	mEq/L	3.0	±1.6	2.9	±1.4	0.3	±0.0	0.3	±0.0	<0.001
	mmol/L	3.0	±1.6	2.9	±1.4	0.3	±0.0	0.3	±0.0	
Electrolytes										
Na^+	mEq/L	145.8	±1.3	146.9	±2.3	146.4	±2.4	148.2	±2.4	0.170
	mmol/L	145.8	±1.3	146.9	±2.3	146.4	±2.4	148.2	±2.4	
K^+	mEq/L	4.2	±0.2	4.1	±0.4	3.6	±0.1	3.6	±0.3	<0.001
	mmol/L	4.2	±0.2	4.1	±0.4	3.6	±0.1	3.6	±0.3	
Ca^{++}	mEq/L	1.1	±0.1	1.1	±0.1	1.1	±0.1	1.1	±0.1	0.307
	mmol/L	0.55	±0.05	0.55	±0.05	0.55	±0.05	0.55	±0.05	
Cl^-	mEq/L	107.4	±3.1	106.6	±2.8	103.9	±2.6	106.3	±2.4	0.046
	mmol/L	107.4	±3.1	106.6	±2.8	103.9	±2.6	106.3	±2.4	
P^-	mEq/L	2.2	±0.4	2.2	±0.5	2.0	±0.5	2.2	±0.5	0.606
	mmol/L	2.2	±0.4	2.2	±0.5	2.0	±0.5	2.2	±0.5	
Proteins										
Albumin	g/dL	4.4	±0.3	4.2	±0.3	3.6	±0.2	3.7	±0.1	<0.001
Globulin	g/dL	1.8	±0.3	1.6	±0.3	1.5	±0.3	1.4	±0.2	0.003
Haematocrit	L/L	0.29	±0.03	0.28	±0.03	0.18	±0.02	0.20	±0.03	<0.001
Haemoglobin	g/dL	9.98	±0.86	9.42	±1.22	6.12	±0.72	6.72	±0.88	<0.001
Independent variables affecting pH										
$PaCO_2$	mmHg	51.5	±8.6	51.2	±6.7	71.4	±15.6	73.8	±9.1	<0.001
SIDa	mEq/L	40.7	±1.9	42.7	±2.7	47.0	±4.3	46.2	±1.4	<0.001
	mmol/L	40.1	±1.9	42.1	±2.7	46.4	±4.3	45.7	±1.6	
SIDe	mEq/L	34.1	±3.0	35.1	±3.6	40.4	±6.0	40.8	±2.0	<0.001
	mmol/L	34.1	±3.0	35.1	±3.6	40.4	±6.0	40.8	±2.0	

Table 2 Measured and calculated values obtained from healthy impala *(Aepyceros melampus)* undergoing immobilisation and general anaesthesia using two different drug protocols *(Continued)*

SIG	mEq/L	6.6	±3.3	7.5	±1.8	6.5	±3.2	5.5	±2.2	0.243
	mmol/L	6.0	±3.2	7.0	±1.4	6.0	±3.2	5.0	±2.2	
AG	mEq/L	13.5	±4.0	14.3	±2.1	10.2	±2.8	9.5	±1.7	<0.001
	mmol/L	13.5	±4.0	14.3	±2.1	10.2	±2.8	9.5	±1.7	
Atot	mmol/L	16.4	±1.2	15.7	±1.2	13.8	±1.1	14.3	±1.0	<0.001

Time1: sampling prior to maintenance of general anaesthesia; Time 2: sampling 1 min prior to ending general anaesthesia; *P-TMP* protocol using thiafentanil-medetomidine immobilisation and propofol-ketamine-medetomidine infusion for general anaesthesia maintenance, *P-EME* protocol using etorphine-medetomidine immobilisation and etorphine-ketamine-medetomidine infusion for general anaesthesia, *P value* level of significance estimated over time, *min* minute, *Resp rate* respiratory rate, *MAP* direct mean arterial blood pressure, HCO_3^- bicarbonate ion, *BE* base excess, Na^+ sodium ion, K^+ potassium ion, Ca^{++} calcium ion, Cl^- chloride ion, P^- phosphorus ion, $PaCO_2$ arterial partial pressure of carbon dioxide, *SIDa* apparent strong ion difference, *SIDe* effective strong ion difference, *SIG* strong ion gap, *AG* anion gap *Atot* total weak acids in plasma, *mmHg* millimetres mercury, *g/dL* grams per decilitre, *mEq/L* milliequivilent per litre, *mg/dL* milligrams per decilitre, *mmol/L* millimoles per litre

and SIDa ($p < 0.001$) increased, while the AG ($p < 0.001$) and Atot ($p < 0.001$) decreased over time. Yet, there was no significant difference between the two drug protocols for $PaCO_2$ ($p = 0.754$), SIDa ($p = 0.552$), AG ($P = 0.963$) and Atot ($p = 0.860$). Overall, the independent variables changed, as follows: $PaCO_2$ from 51.3 ± 7.5 mmHg to 72.6 ± 12.4 mmHg; SIDa from 44.6 ± 2.4 mEq/L to 46.9 ± 3.1 mEq/L; AG from 15.0 ± 3.1 mEq/L to 10.9 ± 2.2 mEq/L; and Atot from 16.1 ± 1.2 mmol/L to 14.0 ± 1.1 mmol/L at Time 1 to Time 2, respectively.

According to the Henderson-Hasselbalch approach, the $PaCO_2$ (already described above) and serum bicarbonate are the variables of interest. Serum bicarbonate increased ($p < 0.001$) and serum lactate decreased ($p < 0.001$) over time, without significant differences between the two protocols at the time points (serum bicarbonate $p = 0.676$; serum lactate $p = 0.782$). Overall, the serum bicarbonate changed from 29.6 ± 2.7 mEq/L to 36.0 ± 4.1 mEq/L; and serum lactate changed from 2.9 ± 1.5 mEq/L to 0.3 ± 0.03 mEq/L at Time 1 to Time 2, respectively.

The blood pH demonstrated a strong negative correlation to the $PaCO_2$ ($r = -0.824$; $p < 0.001$) and a moderate negative correlation to serum bicarbonate ($r = -0.385$; $P = 0.020$). The blood pH did not correlate to the SIDa ($r = -0.316$; $p = 0.060$) and Atot ($r = 0.164$; $p = 0.341$), respectively, but it did correlate moderately and positively to the AG ($r = 0.413$; $p = 0.012$).

The electrolytes did not change over time, with the exception of potassium ($p < 0.001$) and chloride ($p = 0.046$) which both decreased without a significant difference between the two protocols (potassium $p = 0.192$; chloride $p = 0.398$). Overall, potassium changed from 4.1 ± 0.3 mEq/L to 3.6 ± 0.3 mEq/L; and chloride change from 107.0 ± 2.9 mEq/L to 105.1 ± 2.7 mEq/L at Time 1 to Time 2, respectively.

The haematocrit ($p < 0.001$) and haemoglobin concentration decreased ($p < 0.001$) over time, without significant differences between the two protocols at the time points (haematocrit $p = 0.856$; haemoglobin concentration $p = 0.944$). Overall, the haematocrit changed from 0.29 ± 0.03 L/L to 0.19 ± 0.02 L/L; and haemoglobin concentration changed from 9.7 ± 1.1 g/dL to 6.4 ± 0.8 g/dL at Time 1 to Time 2, respectively.

The serum proteins, albumin ($p = <0.001$) and globulin ($p = 0.003$) significantly decreased over time with a significant differences between the two protocols for albumin ($p = 0.036$; interaction: protocol x time), yet, not for globulin ($p = 0.092$; interaction: protocol x time). Overall, the albumin changed from 4.3 ± 0.3 g/dL to 3.6 ± 0.2 g/dL; and globulin changed from 1.7 ± 0.3 g/dL to 1.5 ± 0.2 g/dL at Time 1 to Time 2, respectively.

Discussion

The initial blood pH, after immobilisation, indicated a mild acidaemia (normal ruminant arterial blood pH reference range 7.37 to 7.48 [25]) due to the elevated $PaCO_2$ causing a respiratory acidosis. Thereafter, the $PaCO_2$ increased further and blood pH of the impala significantly decreased over time, regardless of the immobilisation and anaesthetic protocol used. The Henderson-Hasselbalch approach and the quantitative physicochemical Stewart approach were used to interpret the acid-base status of the impala. The profoundly elevated $PaCO_2$ at the end of the anaesthesia would cause a respiratory acidosis, while the rising apparent strong ion difference (SIDa) and waning total weak acids (Atot) contributed to a simultaneous occurring metabolic alkalosis. Furthermore, the progressive elevation of the calculated serum bicarbonate (HCO_3^-) and base excess (BE) both indicate an emerging compensatory metabolic response. Therefore the resultant pH values at the end of the anaesthesia are because of a pronounced metabolic compensatory response to the severe respiratory acidosis that resulted in an overall mild acidaemia. Because there are no published reference ranges for acid-base variables in resting impala we used ranges from closely related species (healthy awake goats and calves; Table 1) to interpret our findings.

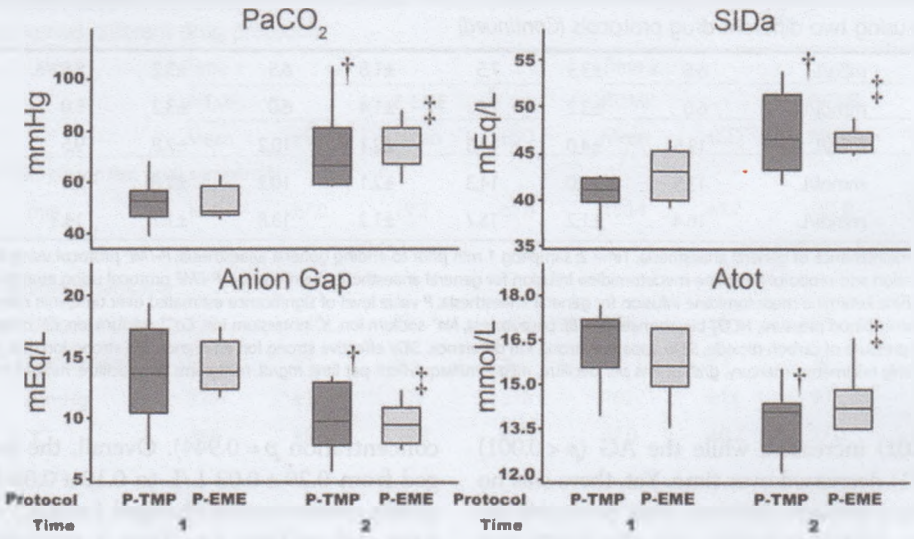

Fig. 1 *Box plot* and whiskers of the independent variables in healthy impala *(Aepyceros melampus)* thought responsible for the change in hydrogen ion concentration (pH) in the plasma. Time 1 was sampling after immobilisation with either thiafentanil-medetomidine (P-TMP) or etorphine-medetomidine (P-EME). Time 2 was sampling at the end of either a propofol-ketamine-medetomidine (P-TMP) or an etorphine-ketamine-medetomidine (P-EME) total intravenous infusion. Where: $PaCO_2$ is the arterial partial pressure of carbon dioxide; SIDa is the apparent strong ion difference; Atot is the total weak acid concentration in plasma; mmHg: millimetres mercury; mEq/L: milliequivence per litre and mmol/L: millimoles per litre; †: significant change in variable value over time for P-TMP protocol; ‡: significant

Unfortunately, more advanced techniques used to calculate compensation, like expected compensatory changes in $PaCO_2$ or bicarbonate ion concentrations, would be difficult to use for interpretation due to the paucity in referenced normal ranges for small wild ungulates. Although the impala were habituated to the boma, they were not tame enough for us to obtain awake control or reference samples. Gaining such samples from an awake wild animal can only be achieved by using remote sampling devices [26]. Such devices are not readily available and need to be custom made per species [27].

We expected respiratory acidosis to be pronounced, especially at the end of the anaesthesia, as $PaCO_2$ was greatly elevated compared to the normal awake range of 35–45 mmHg in mammals [5]. The $PaCO_2$ at the end of the anaesthesia was substantially higher compared to just after induction into immobilisation in our impala, and compared to values measured in other immobilised impala ($PaCO_2$ of 39.1 ± 3.4 to 41.3 ± 5.0 mmHg) [28]. One of the stimuli to take a breath in a healthy awake animal is brought about by the rising $PaCO_2$ level reaching a threshold. The drugs used in this study, especially the potent opioids, either alone or in combination with the other anaesthetic and sedative drugs, are known to cause respiratory-neuronal depression [29, 30] which shifts the carbon dioxide respiratory response curve to the right [31]. Whereby a higher threshold level of $PaCO_2$ is required to stimulate the respiratory centre to initiate a breath. Therefore these drugs ultimately result in hypoventilation (decreased alveolar minute ventilation) which causes the increase in $PaCO_2$. Ventilation is challenging to assess when only subjectively monitoring the respiratory system by counting the respiratory rate and estimating the tidal volume. Often an animal will appear to be ventilating normally, as in the case of these impala that had a normal respiratory rate and tidal volume at Time 1, after dosing with butorphanol (data reported elsewhere) [22, 23], but on closer examination this may not be the case. Thus, more invasive monitoring tools, such as arterial blood gas analysis or capnography, may be required to detect shifts in blood pH that are due to alterations in ventilation [32]. Furthermore, other co-aetiologies should always be considered when there is an obvious respiratory acidosis without overt evidence of simple hypoventilation, such as severe right-to-left pulmonary shunting, large dead-space ventilation or ventilation-perfusion mismatch [5, 23, 30]. Haemoglobin is an important intracellular buffer that will bind reversibly to either carbon dioxide or to the hydrogen ion formed by the bicarbonate buffer system, to transport them from the metabolising tissues to the lungs [4, 5, 8, 33, 34]. With oxygen supplementation an increase in the PaO_2 will increase the force for oxygen to bind to haemoglobin as opposed to carbon dioxide or hydrogen ions (Haldane Effect; high PaO_2 levels decrease the buffering effects of haemoglobin, therefore hydrogen ions are unbound from haemoglobin to preferentially transport oxygen). Therefore, during anaesthesia, the $PaCO_2$ in the impala most likely increased due to

the oxygen supplementation [4, 5, 33, 34]. Furthermore, the haemoglobin concentration (and haematocrit) dropped during general anaesthesia [35], a known phenomenon in patients under general anaesthesia, especially if alpha2-adrenoceptor agonists like medetomidine are used [36], therefore decreasing an important plasma buffering system which could have also contributed to the increase in $PaCO_2$ and hydrogen ion concentration. The emergency treatment of apnoea with butorphanol did result in a regular spontaneous breathing pattern in the impala immobilised with both protocols [22, 23]. A limitation to this study is that we did not take arterial blood samples immediately after recumbency, while apnoeic episodes occurred, especially in P-TMP which required more frequent butorphanol boluses compared to P-EME. Therefore, the effects of butorphanol on the blood pH could not be determined. However, in another etorphine immobilized ungulate, the goat, butorphanol corrected hypoxaemia but not hypercapnia and therefore it may be that it had little influence on blood pH in the impala at Time 1 [37].

Irrespective of the cause of the respiratory acidosis, metabolic compensation, indicated by the significant rise in the bicarbonate ion concentration, occurred within a 120 min. This indicator of compensation is according to the traditional Henderson-Hasselbalch approach used to evaluate blood pH, whereby the body attempts to correct the increased hydrogen ion concentration by elevating the bicarbonate ion concentration to normalise the bicarbonate ion to carbonic acid ratio ($[HCO_3^-]$:$[H_2CO_3]$ ratio) back to 20:1 [4]. The $PaCO_2$ corresponds to H_2CO_3 and is merely substituted to simplify the calculation of the compensatory response [12, 17, 28]. Therefore, any rise in $PaCO_2$ should be met by a rise in the HCO_3^- ions in uncomplicated respiratory acidosis, as demonstrated in these impala.

A simple change in the HCO_3^- does not completely explain the acid-base compensation that occurred in the impala. The apparent strong ion difference (SIDa) was higher and the total weak acids (Atot) were lower than that of published ranges for healthy control goats and calves. Both changes indicate an additional non-respiratory alkalinising effect [5–7, 11]. The measured electrolytes (sodium, potassium, calcium, chloride and phosphorus) were within accepted published ranges for impala [38]. Furthermore, all but the potassium and chloride concentration did not significantly change over time. Yet the decrease in the potassium level was not large enough to solely explain the increased apparent strong ion difference (SIDa) value. Therefore, the decrease in lactate ion concentration that occurred most likely contributed the most to the increased apparent strong ion difference (SIDa) at the 120 min measurement. The drop in the chloride concentration could have been due to the increase in plasma bicarbonate, whereby the plasma attempts to maintain electrical neutrality by excreting chloride [5–7, 12, 39]. At the rate of administration used it is unlikely that the infused physiological saline increased sodium or chloride concentrations in the plasma of the impala, as reported in healthy dogs [40]. The decreased total weak acids (Atot) over time was attributed to the decrease in albumin and globulin concentrations. A decrease in plasma protein levels has been described in animals undergoing general anaesthesia [35], especially when alpha2-adrenoceptor agonists such as medetomidine are administered [36]. The total weak acids (Atot) levels reported in this study were also lower compared to goats and calves. This difference could be attributed to different measurements of phosphorus, or different calculations used to determine the total weak acids (Atot). Furthermore, the rising bicarbonate ion concentration could cause the negatively charged proteins to move out of the plasma in order to maintain electrical neutrality, a plausible theory requiring further confirmation.

The decrease in the anion gap (AG) over time was attributed to the pronounced increase in the HCO_3^- concentration. Overall the anion gap (AG) was substantially lower than those reported for goats and calves [14, 17–20]. However, the HCO_3^- concentrations and BE at the end of the anaesthesia were higher than a generally accepted upper limit of 30 mmol/L and 6.0 to 8.0 mmol/L, respectively for herbivores [4, 5, 8] and the published ranges for healthy goats [14, 17, 19] and calves [18, 20]. Both of these variables demonstrate that a metabolic compensatory response was initiated to correct the respiratory acidosis in the impala.

The worsening acidaemia, and precipitous drop in serum protein concentrations, especially albumin, may alter ionisation and protein binding of drugs, which could have profound effects on drug pharmacokinetics and dynamics [41]. These possible alterations warrant further investigation to gain better clarity of their clinical implications. In other words, did the impala in this study experience more pronounced respiratory depression due to alterations in the pharmacokinetics and dynamics of the drugs used to maintain general anaesthesia by causing a relative overdose?

Furthermore, the clinical implications of acidosis, regardless of cause, are serious and warrant careful consideration. The effects of acidosis on the cardiovascular system include negative inotropy, tachycardia and vasodilation which translates into a decreased blood pressure due to the reduction in cardiac output (decreased stroke volume) and systemic vascular resistance [1, 2]. Oxygen binding to haemoglobin is altered, causing a right shift in the oxygen-haemoglobin

dissociation curve (Bohr Effect; acidaemic plasma pH, usually brought about by increasing $PaCO_2$ levels at metabolically active tissue decrease haemoglobins affinity for oxygen, therefore increase its offloading to the tissue) [4, 5]. The right shift translates into a decrease affinity for haemoglobin to bind to oxygen, therefore less is transported to the tissue potentially resulting in tissue hypoxia. Among other causes, the decrease in pH increases the stimulation to breathe which results in increased workload of the respiratory system and therefore global oxygen demand. Furthermore, the decrease in cardiovascular performance decreases oxygen delivery [2]. Animals that cannot initiate compensatory responses to acidosis due to illness or the effects of anaesthetic drugs or both may suffer further physiological derangements which could lead to increased morbidity or mortality.

The shift in blood pH was evident in healthy impala undergoing immobilisation and general anaesthesia using two different drug protocols. The profound increase in $PaCO_2$, despite a seemingly normal respiratory rate for a medium sized ruminant suggests that monitoring for a respiratory acidosis is not reliable when using just the respiratory rate as an indicator of respiratory suppression. The gases found within the alveoli while breathing room air include nitrogen, oxygen and carbon dioxide (originating from the metabolically active tissue and transported to alveoli via the blood). If the amount of carbon dioxide increases to levels above 50 mmHg, as noted in these impala, then there is an increased competition for gases within the alveoli [4, 5]. This competition decreases the amount of oxygen that is available for absorption and can lead to hypoxaemia [4, 5]. Therefore, if these protocols are to be used in the field, oxygen supplementation should be considered mandatory [22, 23]. Despite the substantial increase in $PaCO_2$ over time the acidaemic shift in the blood pH was negligible due to the profound compensatory metabolic responses that we detected. These important responses require normal health and physiological function and therefore caution should be taken, and acid-base status carefully assessed, when immobilising ill or anorexic wild ungulates.

Conclusion

The profound increase in the partial pressure of carbon dioxide that worsened during the total intravenous anaesthesia in both protocols initiated a substantial metabolic compensatory response to prevent severe acidaemia. This compensation resulted in a clinically acceptable mild acidaemic state, which worsened over time but not between the protocols, in healthy impala. However, these important compensatory mechanisms require normal physiological function and therefore when immobilising ill or anorexic wild ungulates their acid-base status should be carefully assessed. In addition, whenever impala are immobilised with thiafentanil or etorphine based drug combination respiration should be closely monitored and butorphanol and oxygen supplementation should be considered in apnoea and hypoxia occurs.

Abbreviations

AG: Anion gap; Atot: Total weak acids; H^+: Hydrogen ion; HCO_3: Bicarbonate ion; $PaCO_2$: Arterial partial pressure of carbon dioxide; PaO_2: Arterial partial pressure of oxygen; SID: Strong ion difference

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Authors' contributions

GEZ data collection, data analysis, manuscript drafting. LCRM data collection, supervised data analysis, manuscript drafting and editing. All authors read and approved the final manuscript.

Consent for publication

No applicable. Data and applicable material that may be of interest yet not detailed in the manuscript are obtainable form the corresponding author.

Competing interests

The authors declare that they have no competing interests.

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Investigation of c-KIT and Ki67 expression in normal, preneoplastic and neoplastic canine prostate

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Abstract

Background: c-KIT expression has been related to bone metastasis in human prostate cancer, but whether c-KIT expression can be similarly classified in canine prostatic tissue is unknown. This study assessed c-KIT and Ki67 expression in canine prostate cancer (PC). c-KIT gene and protein expression and Ki67 expression were evaluated in forty-four canine prostatic tissues by immunohistochemistry, RT-qPCR and western blot. Additionally, we have investigated c-KIT protein expression by immunoblotting in two primary canine prostate cancer cell lines.

Results: Eleven normal prostates, 12 proliferative inflammatory atrophy (PIA) prostates, 18 PC, 3 metastatic lesions and two prostate cancer cell cultures (PC1 and PC2) were analysed. The prostatic tissue exhibited varying degrees of membranous, cytoplasmic or membranous/cytoplasmic c-KIT staining. Four normal prostates, 4 PIA and 5 prostatic carcinomas showed positive c-KIT expression. No c-KIT immunoexpression was observed in metastases. Canine prostate cancer and PIA samples contained a higher number of Ki67-positive cells compared to normal samples. The median relative quantification (RQ) for c-KIT expression in normal, PIA and prostate cancer and metastatic samples were 0.6 (0.1-2.5), 0.7 (0.09-2.1), 0.7 (0.09-5.1) and 0.1 (0.07-0.6), respectively. A positive correlation between the number of Ki67-positive cells and c-KIT transcript levels was observed in prostate cancer samples. In the cell line, PC1 was negative for c-KIT protein expression, while PC2 was weakly positive.

Conclusion: The present study identified a strong correlation between c-KIT expression and proliferative index, suggesting that c-KIT may influence cell proliferation. Therefore, c-KIT heterogeneous protein expression among the samples (five positive and thirteen negative prostate cancer samples) indicates a personalized approach for canine prostate cancer.

Keywords: CD117 antigen, Dog, Prostatic cancer, Immunohistochemistry, Western blotting

Background

Prostate cancer (PC) in dogs is associated with aggressive tumour behaviour, high metastatic rate and poor prognosis [1]. Bone and lungs are the most common metastatic sites, and 80% of patients show distant metastases at the time of diagnosis [2–4]. Despite the devastation of this disease, few treatment options are currently available for PC-affected dogs [5, 6]. Radiation therapy and non-steroidal anti-inflammatory drugs are considered the best options for metastatic PC [7]. Photodynamic therapy [8], targeted radioiodine imaging [9] and radiolabelled monoclonal antibodies [10] have been experimentally used in pre-clinical canine models of human prostate cancer, but their clinical efficacy is currently unknown. Other targeted therapies have been evaluated, such as inhibition of the c-KIT receptor using toceranib [11, 12]. In fact, recent studies have demonstrated the role of c-KIT in human PC development [13] and metastasis [14].

The proto-oncogene c-KIT belongs to the class III receptor tyrosine kinase family, and its ligand (stem cell factor protein) represents the mast cell growth factor in humans and dogs [15, 16]. In xenografts models of human PC with low c-KIT expression, the receptor is up-regulated during cancer progression through production

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of stem cell factor in the bone microenvironment [14]. Thus, osteoclasts produce stem cell factor, stimulating PC cells to increase c-KIT expression and, subsequently, their migration from the primary tumour to bones [14]. Inhibition of c-KIT using lentiviral short hairpin RNA in human PC cell lines reduced tumour growth and increased the incidence of metastasis, suggesting a key role for c-KIT in intraosseous tumour growth in xenografts model [17].

Moreover, c-KIT has been hypothesized to be involved in neoplastic cell proliferation. In canine mammary tumours, a positive correlation between c-KIT and Ki67 expression has been demonstrated [18]. Brunettti et al. [18] observed a correlation between c-KIT expression and Ki67 immunoexpression, suggesting a correlation between the presence of c-KIT receptor and proliferative activity. These authors indicated that membranous or cytoplasmic immunolocalization promotes proliferation in canine malignant tissue [18]. Our research team evaluated double immunohistochemical expression of c-KIT/Ki67 in canine mast cell tumours [19] and identified a strong correlation between the co-expression of c-KIT and Ki67 with survival time. Thus, patients showing positive immunostaining against both markers (c-KIT/Ki67) experienced decreased survival time [20]. c-KIT expression has been studied in different canine tumours, including mast cell tumours [20], mammary tumours [18], seminomas [19], liposarcomas [19] and gastrointestinal stromal tumours [19]. To the best of our knowledge there is no information about c-KIT expression in canine PC tissue.

Therefore, this study aimed to evaluate c-KIT gene and protein expression in normal prostate, canine preneoplastic lesions (proliferative inflammatory atrophy – [PIA]), PC and its metastasis and correlation with Ki-67 expression and other clinical and biological parameters. Additionally, we investigated c-KIT protein expression in two canine PC primary cell lines.

Methods

Case selection

Forty-four formalin-fixed paraffin-embedded (FFPE) and twelve frozen canine prostatic tissues were selected from the archives of the Veterinary Pathology Service (Univ. Estadual Paulista-UNESP) from 2011 to 2015. Tumour samples were obtained from PC-affected dogs during prostatectomy, surgery or necropsy. Normal prostates and PIA lesions were collected during necropsy from animals that died causes not related to prostatic disease. For western blot analysis, tissue samples were collected and immediately frozen in liquid nitrogen.

All samples were from intact adult dogs. We selected 11 normal prostates, 12 PIA samples, 18 PC and three metastases. The metastatic samples were from three different patients (PC3, PC10 and PC13 – Table 1). The Gleason score of each PC was determined according to Palmieri and Grieco [21], and the histological subtypes were evaluated according to Palmieri et al. [22]. Only PC samples with negative Uproplakin III (UPIII) antibody staining were used. UPIII staining was performed according to Lai et al. [23].

Clinical data

Medical records were assessed to obtain clinical information, treatment modalities, treatment response and outcome for each patient. Clinical data was available for 14 out of 18 PC-affected patients (Additional file 1: Table S1). Radical prostatectomy was the primary treatment for animals with non-metastatic PC (2/14). Three patients (3/14) received metronomic chemotherapy, two patients (2/14) received piroxicam, while one patient (1/14) did not receive any treatment. Metronomic chemotherapy was administered according to Fonseca-Alves et al. [5].

c-KIT and Ki67 protein expression

Slide sections (4 μm) were dewaxed in xylene and rehydrated in ethanol. For antigen retrieval, slides were incubated with citrate buffer (pH 6.0) in a pressure cooker (Pascal®; Dako, Carpinteria, CA, USA) followed by treatment with freshly prepared 3% hydrogen peroxide (code: 2081, Dinamica, Diadema, Brazil) in methanol (code: 956, Dinamica, Diadema, Brazil) for 20 min to inhibit endogenous peroxidase activity and further washed in Tris-buffered saline. Slides were incubated with the following primary antibodies overnight at 4 °C: anti-Ki67 (code: M7240, Monoclonal, mouse anti-human, Clone

Table 1 c-KIT and Ki67 expression in normal prostates and canine prostatic lesions

c-KIT Expression							
	c-KIT Staining		Distribution			Ki67	c-KIT
	Positive	Negative	Membranous	Cytoplasmic	Membr + Cytopl	Median of positive cells	Median of RQ
Normal ($n = 11$)	4	7	2	1	1	1	0.6
PIA ($n = 12$)	4	8	0	4	0	54.5	0.7
PC ($n = 18$)	5	13	0	2	3	366	0.7
Metastasis ($n = 3$)	0	3	0	0	0	578	0.2

PIA Proliferative Inflammatory Atrophy, *PC* prostate cancer, *Membr* membranous, *Cytopl* cytoplasmic

MIB1, Dako, 1:50) and anti-CD117/c-KIT (code: A4502, Polyclonal, rabbit anti-human, Dako, 1:100). Both antibodies have been previously validated against canine tissue [24, 25]. A polymer, peroxidase-based system (code: K4061, Envision, Dako, Carpinteria, CA, USA) was subsequently applied, and 3′-diaminobenzidine tetrahydrochloride (code: K3468, DAB, Dako, Carpinteria, CA, USA) was used as a chromogen for 5 min, followed by Harris haematoxylin (code: 2072, Dinamica, Diadema, Brazil) counterstaining. Negative controls were treated the same except for replacing the primary antibody with Tris-buffered saline. A cutaneous canine mast cell tumour was used as a positive control.

Samples were evaluated according to the percentage of c-KIT-positive expression in neoplastic cells and then classified as c-KIT positive or negative. The following semi-quantitative scores were applied: 0 = negative, 1 = >0–10% positive cells, 2 = 11–40% positive cells, 3 = 41–70% positive cells and 4 = > 70% positive cells. Samples showing a percentage higher than 10% were classified as positive [8]. Distribution of the signal (membranous and/or cytoplasmic) was recorded. For Ki67 analysis, the number of positive neoplastic cells in 10 high power fields (HPF) was calculated.

Primary cell culture

Primary cell lines were previously established in our laboratory. We cultured one c-KIT positive and one c-KIT negative PC (evaluated by immunohistochemistry). Canine PC cells (PC1 and PC2) were cultured in a PEGM™ prostatic medium (Lonza, Basel, Switzerland) at 37 °C in 5% CO2 in culture medium supplemented with 10% inactivated foetal bovine serum (FBS, HYCLONE, Waltham, MA, USA) and 100 U/mL of penicillin G and 100 mg/mL of streptomycin (SIGMA, Portland, OR, USA). Culture medium was discarded and replaced with fresh medium every 48 h. For protein and mRNA extraction, 10^4 cells were cultured in 6-well plates (Inc., Corning, NY, USA) in triplicate. When cell cultures reached greater than 90% confluence, protein and mRNA were extracted.

Protein quantification

Western blotting was performed on normal prostate ($n = 6$), PC samples (n = 6) and the two cell lines. A cutaneous mast cell tumour was used as positive control for c-KIT expression. Tissue samples were mechanically homogenized in 50 mM of Tris–HCl buffer, pH 7.5, 0.25% Triton X-100 and EDTA using the Polytron homogenizer (Kinematica, Lucerne, Switzerland) for 30 s at 4 °C. For both cell lines, RIPA Lysis Buffer (catalog: 89,901, Millipore Co., Bedford, MA, USA) was used for protein extraction following manufacture's recommendation. Then, sample homogenates from prostatic tissue and cell lines were centrifuged. Proteins were extracted from the supernatant and quantified as described by Bradford (1976). Equal amounts of protein (70 μg) obtained from the samples were heated at 95 °C for 5 min in the sample loading buffer and were then subjected to SDS–PAGE separation or electrophoresis under reducing conditions and transferred to nitrocellulose membranes (code: N7892, Sigma Chemical Co., St. Louis, MO). Membranes were blocked with 6% skimmed milk in TBS-T (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 4 h, and the anti-KIT antibody (code: A4502, polyclonal, rabbit-anti human, Dako – CA, USA; 1:300) was applied and incubated overnight. Anti-β-actin antibody (code: sc-1616, polyclonal, goat anti human, Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1:000) was used as a positive control. After incubation with the corresponding horseradish peroxidase-conjugated secondary antibody, the blots were detected by means of chemiluminescence (code: RPN2235, Amersham ECL Select western blotting Detection Reagent, GE Healthcare). Protein bands were quantified by densitometry analysis and expressed as integrated optical density (IOD). c-KIT protein expression was normalised to β-actin. Normalised data are expressed as the mean with standard deviation.

Gene expression

All FFPE prostatic samples were macrodissected using 16-gauge needles, and mRNA were extracted using a commercial RecoverAll™ Total Nucleic Acid Kit (code: AM1975, Ambion, Life Technologies, MA, USA) according to the manufacturer's instructions. mRNA from cell lines was extracted using the RNeasy mini Kit (code: 74,104, Qiagen, Hilden, Germany) following the manufacturer's recommendations. mRNA concentration was determined with a spectrophotometer (NanoDrop™, ND-8000, Thermo Scientific, MA, USA), while mRNA integrity was evaluated with a Bioanalyzer 2100 and an Agilent RNA 6000 Nano Kit (code: 5067-1511, Agilent Technologies, CA, USA). cDNA was synthesized in included in a final volume of 20 μL, with each reaction containing 1 μg of total RNA treated with DNAse I (code: 18,047,019, Life Technologies, Rockville, MD, USA), 200 U of Super Script III reverse transcriptase (code: 18,080,044, Life Technologies, Rockville, MD. USA), 4 μL of 5X Super Script First-Strand Buffer, 1 μL of each dNTP at 10 mM (code: 18,427,088, Life Technologies, Rockville, MD, USA), 1 μL of Oligo-(dT)18 (500 ng/μL) (code: 8,418,012, Life Technologies, Rockville, MD, USA), 1 μL of random hexamers (100 ng/μL) (code: N8080127, Life Technologies, Rockville, MD, USA), and 1 μL of 0.1 M DTT (code: R0861, Life Technologies, Rockville, MD, USA). Reverse transcription was performed for 60 min at 50 °C, and the enzyme was subsequently inactivated for 15 min at 70 °C. cDNA was stored at −80 °C as described by Rivera-Calderón et al. [26].

RT-qPCR for c-KIT (Forward: 5′-CCAGTGTGTG GTTGCAGGAT-3′ and Reverse: 5′-CTCAGCTCCTGG ACAGAAATACC-3′) and the endogenous genes *HPRT* (Forward: 5′-AGCTTGCTGGTGAAAAGGAC-3′ and Reverse: 5′-TTATAGTCAAGGGCATATCC-3′), *ACTB* (Forward: 5′-GGCATCCTGACCCTCAAGTA-3′ and Reverse: 5′-CTTCTCCATGTCGTCCCAGT-3′) and *RPS5* (Forward: 5′-TCACTGGTGAGAACCCCCT-3′ and Reverse: 5′-CCTGATTCACACGGCGTAG-3′) was conducted in a total volume of 10 μL containing Power SYBR Green PCR Master Mix (Applied Biosystems; Foster City, CA, USA), 1 μL of cDNA (1:10) and 0.3 μL of each primer. Reactions were performed in triplicate in 384-well plates using QuantStudio 12 K Flex Thermal Cycler equipment (Applied Biosystems; Foster City, CA, USA). A dissociation curve was included in all experiments to determine the PCR product specificity. Relative gene expression was quantified using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

All groups (normal, PIA, PC and metastases) were evaluated as the median, and an analysis of variance (ANOVA) was used to evaluate any difference among the groups. Data are presented as the mean ± SD. A t test was used to verify the significant difference in Ki67 expression and c-KIT gene expression. The survival curve was calculated only for PCs using the Kaplan-Meier method, and the statistical significance was determined using a log-rank test. The overall survival was defined as the period (in months) between the date of surgery and death. We evaluated the overall survival of all patients according to the chemotherapy protocol, c-KIT expression (c-KIT transcript level and immunoexpression) and Ki67 expression (low Ki67 expression or high Ki67 expression). The Ki67-positive samples were categorized as low or high according to the median expression. A t test was used for western blotting analysis. A t test was applied to analyse the c-KIT transcript levels comparing two variables. $P < 0.05$ was considered significant for all variables. Analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA).

Results

Clinical data

All clinical information about breed, age and metastatic history from the fourteen dogs with PC are shown in Additional file 1: Table S1. Based on clinical records, the outcome was identified in 8 out of 14 (57%) patients. The median survival time was 165 days (12 – 423 days). Despite the heterogeneous survival rate due to different treatments, the patients that received metronomic chemotherapy experienced the highest survival time ($p = 0.01$). There were no significant differences between survival time and c-KIT or Ki67 expression. Based on the Gleason score classification, 10 patients showed a tumour with a Gleason score 10 (10/14), two patients showed a Gleason score 6 (2/14), one patient presented a Gleason Score 8 (1/14) and the other patient showed a Gleason Score 9 (1/14). Seven patients (7/14) showed metastases, primarily to lungs and bones. Four patients with metastases (4/7) showed PC with a Gleason score 10, one patient (1/7) had Gleason score 8 and one had a Gleason score 6 (1/7).

Histology and growth pattern

The cribriform growth pattern was the most common histological subtype of PC (8/18), followed by the solid (5/18), small acinar (2/18), papillary (1/18) and signet ring (1/18). Only one sample (1/18) showed a mixed pattern (small acinar and cribriform). All histological subtypes are summarized in Additional file 1: Table S1 and Fig. 1. Regarding the co-existence of preneoplastic and neoplastic lesions, PIA lesions were observed surrounding carcinomas in 66.7% (12/18) of PC samples.

c-KIT and Ki67 protein expression

No correlation between histological subtype and c-KIT expression was identified. However, all PC samples positive for c-KIT expression showed a Gleason score 10.

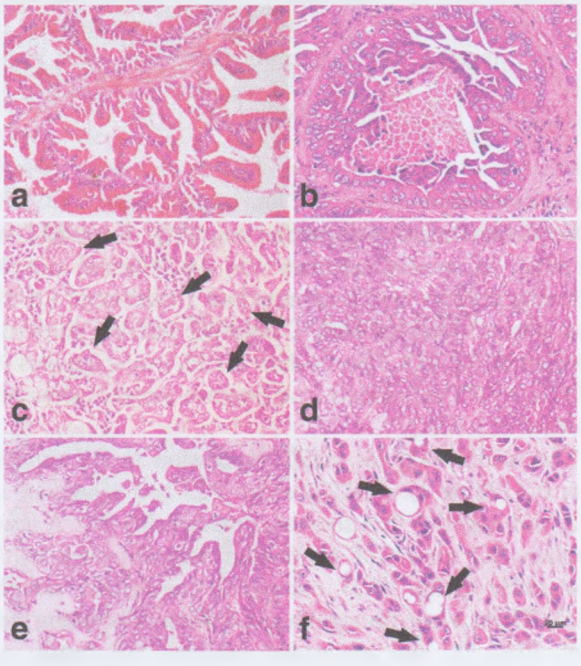

Fig. 1 Histological evaluation of canine prostatic tissue. **a** Histological appearance of a normal prostate gland showing cuboid epithelial cells arranged in an acinar pattern. **b** Canine prostate cancer (PC) showing a cribriform pattern with central comedonecrosis (Gleason Score 10). **c** Canine PC presenting a small acinar pattern (black arrows) (Gleason score 6). **d** Histological appearance of a canine PC with a solid pattern (Gleason Score 10). **e** Canine PC showing a papillary pattern (Gleason Score 8). **f** Canine PC showing a signet ring (black arrows) histological pattern (Gleason Score 10)

The immunohistochemical data are presented in Table 2. A total of 36.4% of normal samples (4/11) showed positive c-KIT staining (Fig. 2), and 33.3% (4/12) and 27.7% (5/18) of PIA and PC samples were c-KIT positive (Fig. 2). All metastases (3/3 – 100%) were c-KIT-negative. Regarding the semi-quantitative immunohistochemical analysis, 50% of c-KIT-positive normal samples (2/4) showed a score of 4 and 50% (2/4) showed a score of 3, while one c-KIT-positive PIA sample (1/4) had a score of 2 and the other three had scores of 3 (3/4). Two (2/5) c-KIT-positive PC samples had scores of 3, two (2/5) had scores of 2, and one (1/5) had a score of 4. Positive c-KIT staining was also observed in stromal cells (Fig. 2).

PIA and PC samples contained higher numbers of Ki67-positive cells (Fig. 2) than did normal samples (Table 1). A progressive increase in the number of positive cells was observed in PIA, PC and metastatic samples ($p < 0.0001$). The mean number of positive cells in normal, PIA, PC and metastatic samples was 1.0, 54.5, 366 and 578, respectively (Table 1).

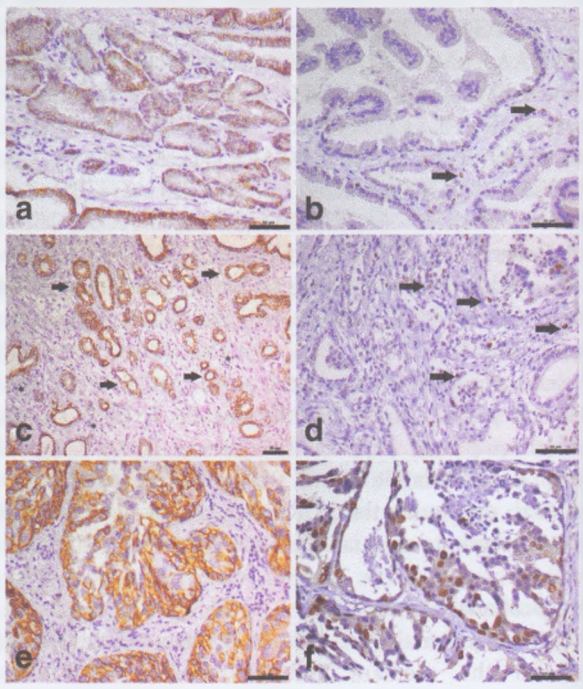

Fig. 2 Immunohistochemical analysis of canine prostatic tissue for c-KIT and Ki67. **a** Normal canine prostate. Normal epithelial cells showing specific membranous staining (black arrow). **b** Ki67 expression in canine prostatic tissue. Arrows indicate positive nuclear expression in basal and stromal cells and no expression in luminal cells. **c** c-KIT expression in PIA lesion. Epithelial atrophic cells showed membranous and cytoplasmic c-KIT staining (arrows). **d** Ki67 expression in PIA lesion. There is positive nuclear expression in basal epithelial atrophic cells. **e** Canine prostate cancer. Neoplastic cells show membranous and cytoplasmic staining. Few c-KIT-positive stromal cells are observed. **f** Ki67 expression in canine prostate cancer. Note a high number of neoplastic cells showing nuclear expression. DAB chromogen, Harris haematoxylin counterstain. 200×

Protein quantification

Two bands of 100 and 155 KDa were identified in canine prostatic tissue. Normal and PC samples showed a similar pattern of c-KIT expression with no significant difference between the two groups (Fig. 3). Three samples (3/6) with negative c-KIT immunoexpression were evaluated by western blotting, and we identified positive expression for both bands (100 and 155 KDa).

Gene expression

The median relative quantification (RQ) for c-KIT expression in normal samples was 0.6. Normal samples showing positive c-KIT staining had a median of 1.6 RQ, and normal samples with a negative staining showed a median of 0.5 RQ. The median for PIA samples was 0.7 RQ for *c-KIT* expression, and positive samples showed a median of 1.3 RQ.

The median RQ for *c-KIT* expression in PC samples was 0.7 (Table 1). c-KIT-positive PCs had a median 1.5 RQ, and negative PCs showed a median 0.5 RQ. A lower median RQ for *c-KIT* expression was observed in the metastatic group (0.1) compared to normal samples (0.6). There were no significant differences among normal, PIA, PC and metastatic samples. All results are shown in Table 1, Table 2 and Fig. 4.

A positive correlation between the number of Ki67-positive cells and c-KIT relative expression was identified (Spearman $R = 0.8287$; $P < 0.0001$). Tumours showing the highest *c-KIT* RQ had the highest number of Ki67-positive cells (Fig. 5).

Primary cell culture

We grew both primary cell lines at passage 10. The PC1 cell line is from a primary tumour showing no c-KIT immunoexpression and presenting RQ 0.091 in RT-qPCR analysis. PC1 cells had no c-KIT protein expression (Fig. 6) and presented no detectable c-KIT

Table 2 Median of c-KIT gene expression and number of Ki67-positive cells in normal prostates and canine prostatic lesions according to c-KIT positive or negative immunostaining

	Normal c-KIT positive samples	Normal c-KIT negative samples	c-KIT positive PIA	c-KIT negative PIA	c-KIT positive PC	c-KIT negative PC	c-KIT negative metastasis
c-KIT gene Expression	1.6 (1.0-2.5)	0.6 (0.15-0.7)	1.3 (0.7-2.1)	0.2 (0.09-0.8)	1.5 (1.2-5.1)	0.5 (0.09-0.9)	0.1 (0.06-0.6)
Ki67-positive cells	2 (1-3)	0 (0-1)	75.5 (37-99)	49 (23-105)	645 (458-751)	301 (109-508)	578 (453-611)

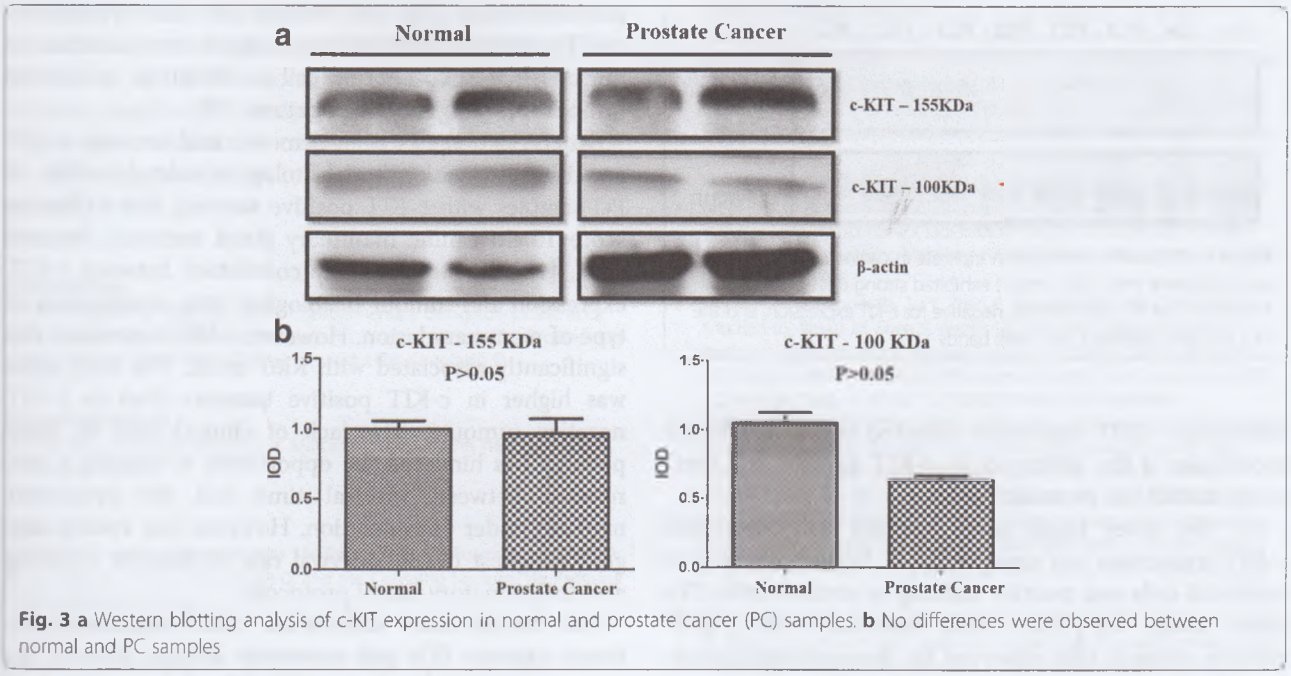

Fig. 3 **a** Western blotting analysis of c-KIT expression in normal and prostate cancer (PC) samples. **b** No differences were observed between normal and PC samples

transcript. The PC2 cell line is from a primary PC with c-KIT membranous and cytoplasmic immunoexpression and had 1.429 RQ by RT-qPCR analysis. The PC2 cells had very low c-KIT protein expression (Fig. 6) compared to controls (mast cell tumour) and showed 0.986 RQ by RT-qPCR.

Discussion

We evaluated c-KIT protein and gene expression in canine prostatic tissue and found a small number of samples were positive for *c-KIT*. Our western blotting results identified positive protein expression in all samples analysed (6 normal and 6 PC), including three samples with KIT negative immunostaining. All prostatic samples contained transcript levels of c-KIT; however, only PCs with relative quantification higher than 1.211 (5/18) demonstrated positive c-KIT immunoexpression. Protein expression is regulated by a complex process involving DNA transcription, epigenetic modification and mRNA degradation [27], and protein expression is directly related to mRNA degradation and protein half-life [28]. Thus, changes and regulation of protein expression occur at multiple levels. One hypothesis for the

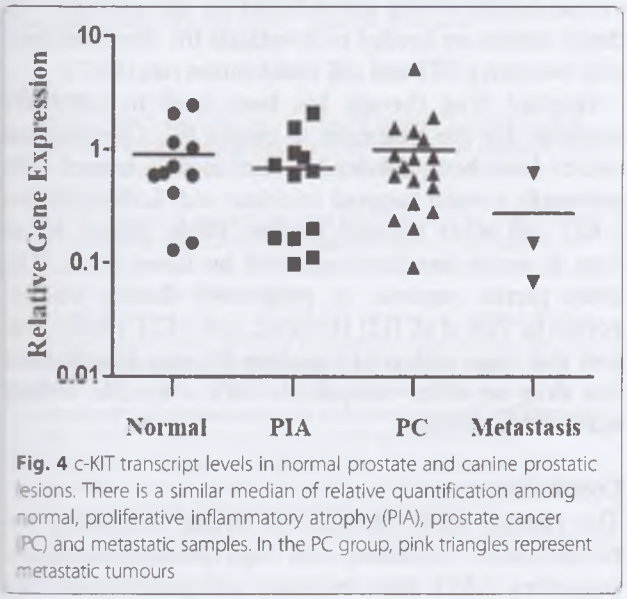

Fig. 4 c-KIT transcript levels in normal prostate and canine prostatic lesions. There is a similar median of relative quantification among normal, proliferative inflammatory atrophy (PIA), prostate cancer (PC) and metastatic samples. In the PC group, pink triangles represent metastatic tumours

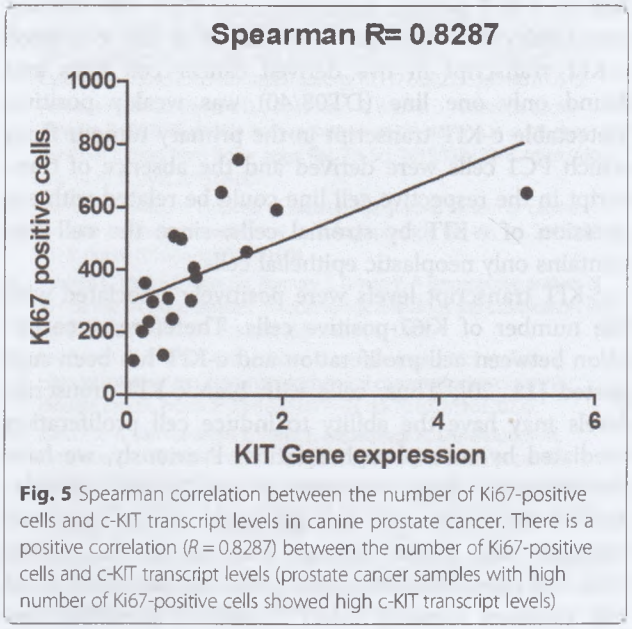

Fig. 5 Spearman correlation between the number of Ki67-positive cells and c-KIT transcript levels in canine prostate cancer. There is a positive correlation ($R = 0.8287$) between the number of Ki67-positive cells and c-KIT transcript levels (prostate cancer samples with high number of Ki67-positive cells showed high c-KIT transcript levels)

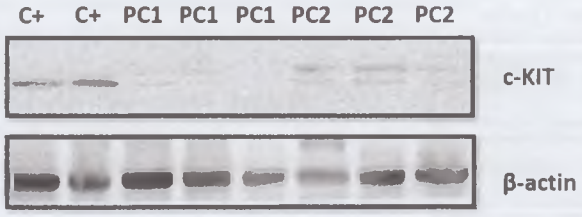

Fig. 6 c-KIT protein expression in triplicate in canine cell lines. Positive control (canine mast cell tumour) exhibited strong c-KIT expression (100 KDa). The PC1 cell line was negative for c-KIT expression, and the PC2 cell line presented two weak bands

differential c-KIT expression detected between different techniques is the difference in c-KIT protein and transcript stability in prostatic tissue.

On the other hand, some samples with detectible c-KIT transcripts had negative c-KIT immunostaining in epithelial cells and positive staining in stromal cells. The latter results were most likely associated with c-KIT-positive stromal cells observed by immunohistochemistry. This result may suggest that stromal cells play an important role in tissue microenvironment maintenance. The levels of protein and transcript detected by western blotting and RT-qPCR in samples showing c-KIT negative immunoexpression by epithelial cells could indicate that other cell types show c-KIT expression in these cases. These samples showed Ki67 expression only in basal and/or stromal cells, indicating a role for cell proliferation in c-KIT expression.

To evaluate c-KIT inhibition in vitro, we cultured two canine PC cell lines (one c-KIT positive and other c-KIT negative by immunohistochemistry), and evaluated protein expression by western blotting. PC1 cancer cells had no c-KIT protein expression, and PC2 cells showed very weak c-KIT expression. Moulay et al. [29] evaluated c-KIT transcript in five derived cancer cell lines and found only one line (DT08/40) was weakly positive. Detectable c-KIT transcript in the primary tumour from which PC1 cells were derived and the absence of transcript in the respective cell line could be related with expression of c-KIT by stromal cells, since the cell line contains only neoplastic epithelial cells.

c-KIT transcript levels were positively associated with the number of Ki67-positive cells. Therefore, a correlation between cell proliferation and c-KIT has been suggested [18. 20]. Thus, cells with high c-KIT transcript levels may have the ability to induce cell proliferation mediated by auto phosphorylation. Previously, we have demonstrated high number of c-KIT/Ki67 double-stained neoplastic cells in high-grade canine mast cell tumours [20]. These tumours showing double stained cells had internalization of the c-KIT receptor [20]. Mast cell tumours showing c-KIT I pattern presented few neoplastic mast cells with nuclear Ki67 and cytoplasmic c-KIT pattern. These findings suggest internalization of the c-KIT receptor during cell proliferation, at least in tumours showing a c-KIT I pattern [20].

No correlation has been demonstrated between c-KIT and Ki67 expression and histological subtype, while all PC samples with c-KIT positive staining had a Gleason score 10. In canine mammary gland tumours, Brunetti et al. [18] did not find any correlation between c-KIT expression and tumour histological type, invasiveness or type of mammary lesion. However, c-KIT expression was significantly associated with Ki67 index. The Ki67 value was higher in c-KIT positive tumours than in c-KIT negative tumours. The lack of clinical data in some patients has hindered the opportunity to identify a correlation between survival time and the prognostic markers under consideration. However, our results suggest a highest overall survival rate in patients receiving anti-inflammatory-based protocols.

Our results show differential c-KIT expression between primary PCs and metastatic lesions. Primary tumours showed a median c-KIT RQ of 0.7, while their corresponding metastases demonstrated lower transcript levels (median 0.1). Therefore, c-KIT may not be associated with the metastatic process in canine PCs. Interestingly, two (2/3) metastases were in bone, and human studies have shown that *c-KIT* signalling is associated with bone metastasis [14, 17]. Bone metastasis in human PC is strongly correlated with c-KIT and SCF signalling [14, 17]. A previous study using a xenograph model proposed that the bone microenvironment expresses SCF to induce expression of c-KIT by neoplastic epithelial cells, acting as an attractant for neoplastic cell migration [14]. It should be noted that this study has a limited number of samples, and there is a need to assess more samples from various breeds of dog and different age groups. More in-depth studies are needed to investigate the direct relationship between c-KIT and cell proliferation rate (Ki67).

Targeted drug therapy has been used in veterinary medicine for the treatment of canine PC. Controversial results have been obtained in canine PCs treated with toceranib, a multi targeted inhibitor with activity against c-KIT and other tyrosine kinases; stable disease for at least 6 weeks has been reported by Chon et al. [11], while partial response or progressive disease was reported by Pan et al. [12] However, our c-KIT results suggest that dogs with c-KIT-positive PC may benefit from this drug or other compounds with a specific activity against this receptor.

Conclusions

The present study identified a strong correlation between c-KIT expression and high proliferative index. suggesting c-KIT may influence cell proliferation. We

identified no relationship between c-KIT expression and metastatic tumours. Therefore, heterogeneous protein expression of c-KIT among samples (five positive and thirteen negative PC samples) indicates a personalized approach is needed for canine PC. Thus, each canine PC should be evaluated individually for more accurate patient care.

Abbreviations
ANOVA: Analysis of variance; cDNA: Complementary deoxyribonucleic acid; FFPE: Formalin-fixed paraffin-embedded; PC: Prostate cancer; PIA: Proliferative inflammatory atrophy; RNA: Ribonucleic acid; UPIII: Uroplakin III

Acknowledgements
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Authors' contributions
The experimental design and data analyses were performed by CEFA, PEK, CP and RLA. CEFA and PEK performed immunohistochemical, RT-qPCR and western blotting experiments. RLA supervised all histological and molecular procedures. CEFA wrote the manuscript draft. All authors critically reviewed the draft and approved the final manuscript.

Consent for publication
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The authors declare that they have no competing interests.

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Abbreviations

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Authors' contributions

The experimental design and data analyses were performed by CEFA, PEK, CF and RLA. CEFA and PEK performed immunohistochemical, RT-qPCR and western blotting experiments. RLA supervised all histological and molecular procedures. CEFA wrote the manuscript draft. All authors critically reviewed the draft and approved the final manuscript.

Consent for publication

Not applicable

Competing interests

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Sponsorship bias and quality of randomised controlled trials in veterinary medicine

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Abstract

Background: Randomised controlled trials (RCTs) are considered the gold standard form of evidence for assessing treatment efficacy, but many factors can influence their reliability including methodological quality, reporting quality and funding source.
The aim of this study was to examine the relationship between funding source and positive outcome reporting in veterinary RCTs published in 2011 and to assess the risk of bias in the RCTs identified.

Methods: A structured search of PubMed was used to identify feline, canine, equine, bovine and ovine clinical trials examining the efficacy of pharmaceutical interventions published in 2011. Funding source and outcomes were extracted from each RCT and an assessment of risk of bias made using the Cochrane risk of bias tool.

Results: Literature searches returned 972 papers, with 86 papers (comprising 126 individual RCTs) included in the analysis. There was found to be a significantly higher proportion of positive outcomes reported in the pharmaceutical funding group (P) compared to the non-pharmaceutical (NP) and 'no funding source stated' (NF) groups (P = 56.9%, NP = 34.9%, NF = 29.1%, $p < 0.05$). A high proportion of trials had an unclear risk of bias across the five criteria examined.

Conclusions: We found evidence that veterinary RCTs were more likely to report positive outcomes if they have pharmaceutical industry funding or involvement. Consistently poor reporting of trials, including non-identification of funding source, was found which hinders the use of the available evidence.

Keywords: Clinical trials, Study design and data analysis, Evidence based medicine, Risk of bias

Background

In order to effectively practice veterinary medicine in an evidence-based way, it is imperative that accurate scientific evidence is available so that the evidence base is complete, reliable, and therefore not misleading. Randomised controlled trials (RCTs), along with their synthesis in the form of systematic reviews, are considered to be the gold standard method for assessing the efficacy of treatment interventions and are a valuable source of information on which to base clinical decisions [1]. The results of RCTs can however be affected by many biases including selection, performance, detection, attrition and reporting biases [2, 3]. The presence of bias can lead to misinterpretation of treatment efficacy or harms, and mislead clinicians when putting the evidence into practice.

Sponsorship bias (the influence of funding source on the reporting of trial results) is an additional potential problem when assessing the reliability of RCTs. The medical literature contains differing reports over whether financial conflicts of interest influence the reported results of a trial. Some studies report a greater likelihood of positive results for industry funded trials [4, 5], while some report no difference between industry and non-industry sponsored trials [6, 7]. A recent overview of medical literature in a Cochrane systematic review concluded that drug and medical device studies were more likely to report favourable results when the study was sponsored by a manufacturer [8].

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There have been several studies examining the methodological and reporting quality of clinical trials in the published veterinary literature [9–11]. Such studies have highlighted issues with the reporting of RCTs and have shown how these reporting deficiencies are associated with an increased likelihood of a trial reporting one or more positive outcomes [10]. To our knowledge, no studies to date have examined the influence of funding source on the likelihood of reporting positive outcomes in the veterinary RCT literature.

The aim of this study was to examine the relationship between funding source and proportions of positive outcome reporting in veterinary RCTs involving a pharmaceutical intervention published in a single calendar year (2011). A secondary aim was to assess the risk of bias of veterinary RCTs published in the same time period.

Methods

A cross-sectional study of veterinary RCTs was conducted. The target population was feline, canine, equine, bovine and ovine RCTs where a pharmaceutical agent was the intervention of interest and efficacy was assessed. The sample population was feline, canine, equine, bovine and ovine RCTs published in 2011 within journals indexed in PubMed.

Search strategy and filtering of results

A structured search of PubMed was conducted in June 2013 using the "clinical trial" Publication Type combined with the relevant species MeSH heading e.g. "clinical trial" [publication type] AND cats [mh]. This was done for each of the 5 species studied: cats, dogs, horses, cattle and sheep (Fig. 1). The search was limited to one calendar year with a PubMed filter: 01/01/11–31/12/11. Search results were exported into EndNote® software for filtering. Papers indexed as RCTs by PubMed ("randomised controlled trials" [publication type]) were extracted, investigators then confirmed if they were RCTs according to the Cochrane definition below (http://www.cochrane.org/glossary/):

"An experiment in which two or more interventions, possibly including a control intervention or no intervention, are compared by being randomly allocated to participants. In most trials one intervention is assigned to each individual but sometimes assignment is to defined groups of individuals (for example, in a household) or interventions are assigned within individuals (for example, in different orders or to different parts of the body)."All publications containing trials confirmed by the investigators as being RCTs, published in 2011, and relevant to the species of interest were then categorised into four intervention subcategories based on the main intervention of interest of the study (Table 1 - Level 1 exclusion criteria):

1. Pharmaceutical – consisting of an active pharmaceutical ingredient, including anthelmintics and vaccines
2. Nutritional
3. Para-pharmaceutical – including probiotics, prebiotics, synbiotics, nutraceuticals and supplements/vitamins/minerals if not considered part of the total dietary ration
4. Other – including surgical interventions, management/husbandry interventions, non-medicinal shampoos, studies relating to diagnostic tests.

Only publications within the 'Pharmaceutical intervention' subcategory were included in this study; these were assessed for further eligibility for analysis according to the second level of inclusion and exclusion criteria in Table 1.

Publications included in the analysis were therefore single dose efficacy studies of pharmaceutical interventions in cats, dogs, horses, cattle or sheep published in 2011. In the case of a publication containing more than one trial, each trial was included independently in the analysis if it met all inclusion criteria.

Sources of funding

For each included trial the source of funding was categorised as one of the following:

1. Pharmaceutical company funding stated or pharmaceutical company involvement (e.g. drug donated by a pharmaceutical company or authors associated with a pharmaceutical company) (P)
2. Non-pharmaceutical company funding stated (NP)
3. No funding source stated (NF)

Outcome recording

All outcomes mentioned in the methods section of the manuscripts were extracted and the result for each outcome was recorded. Outcomes that were reported as results but not mentioned in the methods were not included in the analysis. The result for each outcome was recorded in one of the five categories below (adapted from [10]):

1. Treatment of interest had a statistically significant positive effect on the outcome
   - Treatment better than any control group
   - Treatment equal to positive control group (whether non-inferiority/equivalence design or not)
   - Safety/lack of adverse effects equal to, or better than, any control group

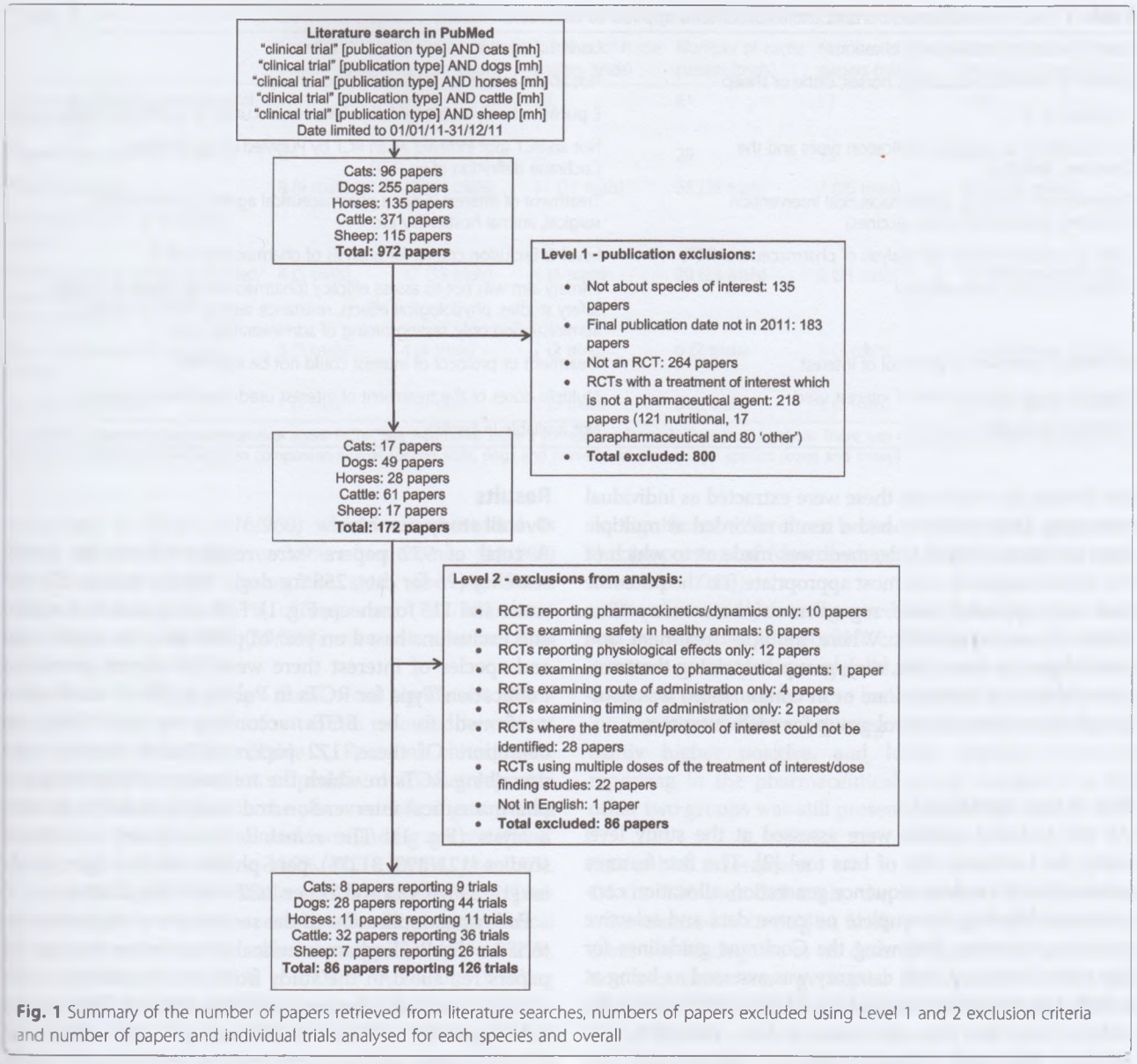

Fig. 1 Summary of the number of papers retrieved from literature searches, numbers of papers excluded using Level 1 and 2 exclusion criteria and number of papers and individual trials analysed for each species and overall

2. Treatment of interest had a statistically significant negative effect on the outcome
   - Treatment worse than any control group
   - Treatment equal to negative control group
   - Safety/adverse effects worse than any control group
   - Treatment equal to a positive control group in a superiority analysis

3. No significant difference between treatment and control groups
   - Outcome remained constant throughout the study (no measurable effect of treatment on the outcome)

4. Results for the outcome were described only
   - There was data reported for an outcome that could have been statistically analysed, but no analysis was presented (if an outcome did not occur in any group, e.g. adverse events, it was treated as having been statistically analysed)
   - Outcomes such as descriptions of pathological appearances with no numerical data attached.

5. Results for the outcome were not reported

Outcome measures that had multiple components (e.g. complete blood count and serum biochemistry, meat yield and meat quality grade assessments) were classed as a single outcome each unless specific features were relevant to

Table 1 Two levels of inclusion and exclusion criteria applied to the search results

Level 1: Inclusion criteria for publications	Level 1: Exclusion criteria for publications
Species of interest is cats, dogs, horses, cattle or sheep	Not about cats, dogs, horses, cattle or sheep
Published in 2011	E published only in 2011 if full publication occurred in a different calendar year
RCT according to PubMed publication types and the Cochrane definition	Not an RCT (not indexed as an RCT by PubMed or not fulfilling Cochrane definition of an RCT)
Treatment of interest is a pharmaceutical intervention (including anthelmintics and vaccines)	Treatment of interest is not a pharmaceutical agent e.g. nutritional, surgical, animal husbandry etc
Level 2: Inclusion criteria for analysis of pharmaceutical RCTs	Level 2: Exclusion criteria for analysis of pharmaceutical RCTs
Primary aim is to assess efficacy	Primary aim was not to assess efficacy (pharmacokinetic/dynamic studies, safety studies, physiological effects, resistance testing, testing routes of administration only, testing timing of administration only)
Identifiable treatment or protocol of interest	Treatment or protocol of interest could not be identified
Single dose of the treatment of interest used	Multiple doses of the treatment of interest used/dose finding studies
Published in English	Not available in English

the disease, in which case these were extracted as individual outcomes. If an outcome had a result recorded at multiple time points, an overall judgement was made as to which of the above categories was most appropriate (i.e. the outcome was only recorded once regardless of how many time points it was measured). Where multiple treatment and control groups were used, each group containing the treatment of interest (either alone or in combination) was compared to its relevant control group for each outcome.

Risk of bias assessment

All the included studies were assessed at the study level using the Cochrane risk of bias tool [2]. The five features assessed were: random sequence generation, allocation concealment, blinding, incomplete outcome data and selective outcome reporting. Following the Cochrane guidelines for the risk of bias tool each category was assessed as being at a high, low or unclear risk of bias. These features allow the risks of selection bias, performance bias, detection bias, attrition bias and reporting bias to be assessed (see Additional file 1 for definitions of these types of bias). We did not include the category of 'Other bias' from the tool.

All assessments made throughout the study were agreed upon by two authors (KW and RH/RD) with any disputes resolved by a third author (RD/RH).

Statistical analysis

Categorical data were presented descriptively as raw numbers and percentages. Associations between funding source and positive outcome reporting were analysed using a Pearson's chi squared test and Bonferroni post hoc test with adjusted p values. Significance level was set at $p < 0.05$. Results for different species are described only and were not compared statistically due to small group sizes. All statistical analyses were conducted in IBM SPSS Version 21.

Results

Overall study numbers

A total of 972 papers were retrieved from the initial searches (96 for cats, 255 for dogs, 135 for horses, 371 for cattle and 115 for sheep; Fig. 1). Following an initial review and exclusions based on year of publication in paper copy and species of interest there were 410 papers given the Publication Type for RCTs in PubMed; 390 of which were confirmed to be RCTs according to the Cochrane definition. Of these, 172 papers (172/390, 44.1%) were describing RCTs in which the treatment of interest was a pharmaceutical intervention and were included in further analysis (Fig. 1). The remainder comprised nutritional studies (121/390, 31.0%), para-pharmaceutical agent studies (17/390, 4.4%) and 'other RCTs' (80/390, 20.5%).

Following application of the second set of exclusion criteria to the RCT pharmaceutical intervention studies, 86 papers remained in the study from which outcomes, bias and sources of funding were extracted (Fig. 1, Table 2 and Additional file 2: Table S1). Eleven papers (all except one of which were within the pharmaceutical funding group) reported more than one RCT, notably one sheep paper reported 19 separate RCTs. As each trial was assessed individually as a separate entry, there were 126 trials included in the full analysis (Table 2 and Additional file 3 for full references of the publications analysed).

Of these 126 trials, 86 (68.3%) were funded by the pharmaceutical industry or had pharmaceutical company involvement, 19 trials (15.1%) explicitly stated they were not funded by the pharmaceutical industry, and 21 trials (16.7%) did not state any source of funding within the manuscript (Table 2).

Funding source and outcome reporting

From the 126 trials included in the analysis, a total of 960 outcomes were extracted. Overall, 47.5% of outcomes (456/960) recorded in the trials were statistically positive

Table 2 Number and funding source of papers and individual trials following level 2 exclusion criteria application

	Number of cat papers (trials)	Number of dog papers (trials)	Number of horse papers (trials)	Number of cattle papers (trials)	Number of sheep papers (trials)	Total number of papers (trials, % of total trials)
Papers including pharmaceutical agent RCTs	17	49	28	61	17	**172**
Papers excluded from analysis[a]	9	21	17	29	10	**86**
Papers analysed	8 (9 trials)	28 (44 trials)	11 (11 trials)	32 (36 trials)	7 (26 trials)	**86 (126 trials)**
Funding sources of analysed papers						
Pharmaceutical company funded/ pharmaceutical company involvement	4 (5 trials)	17 (33 trials)	4 (4 trials)	20 (23 trials)	2 (21 trials)	**47 (86 trials; 68.3%)**
Non-pharmaceutical funding stated	3 (3 trials)	4 (4 trials)	2 (2 trials)	6 (7 trials)	3 (3 trials)	**18 (19 trials; 15.1%)**
No funding stated	1 (1 trial)	7 (7 trials)	5 (5 trials)	6 (6 trials)	2 (2 trials)	**21 (21 trials; 16.7%)**

Included studies are the pharmaceutical agent RCTs. [a]See Additional Table 1 for reasons for exclusions from analysis. There was no statistical difference ($p = 0.53$) between funding sources between companion animal species (cats, dogs and horses) and farm animal species (cows and sheep)

compared to 28.8% (276/960) which were recorded as being statistically negative; 1.9% of outcomes (18/960) remained unchanged during the study (no significant difference category), 14.7% of outcomes (141/960) were described only and 7.2% (69/960) were not reported at all in the results (Table 3).

Between funding groups there were significant differences in the proportions of outcomes recorded in each of the outcome categories (Table 3, Pearsons chi squared, $p < 0.001$). The proportion of positive outcomes reported was significantly higher in the pharmaceutical group than in the non-pharmaceutical and 'no funding source stated' groups ($P = 56.9\%$, NP = 34.9%, NF = 29.1%, $p < 0.05$). Correspondingly, there was a significantly lower proportion of negative outcomes recorded for the pharmaceutical group compared to the other two groups ($P = 23.5\%$, NP = 37.6%, NF = 37.1%, $p < 0.05$). Across all funding groups the proportion of outcomes recorded as 'no significant difference' was low, however the 'no funding group' had a significantly higher proportion compared to the pharmaceutical group (NF = 4.6%, $P = 0.8\%$, $p < 0.05$); the non-pharmaceutical group was not different to either of the other two groups (NP = 2.6%, $p > 0.05$). There were no significant differences between the funding groups in the proportion of 'described only' or 'not reported' outcomes ($p > 0.05$).

The above analysis categorised a treatment group which had equal results to a positive control group as a 'positive' outcome, even if the study did not use a non-inferiority design. If these results were instead considered to be in a 'no significant difference' category, the pattern of significantly higher positive, and lower negative, outcome reporting in the pharmaceutical group compared to the other two groups was still present ($p < 0.05$).

Risk of bias assessment

Of the 126 included trials, the majority (92/126, 73.0%) were assessed as having an unclear risk of selection bias as there was inadequate or no description of how randomisation sequences were generated and employed. The vast majority of the trials were assessed as having an unclear risk of bias for allocation concealment (109/126, 86.5%) as it was impossible to determine what procedures had been followed. Blinding was reported more consistently, with 44 of the 126 trials (34.9%) being assessed as having a low risk of bias, 72/126 (57.1%) having an unclear risk, and the

Table 3 Categorisation of individual outcomes from 126 trials (960 outcomes)

	Outcomes from trials with pharmaceutical funding/involvement	Outcomes from trials with non-pharmaceutical funding stated	Outcomes from trials with no funding source stated	Outcomes from all trials
Positive outcomes	56.9% $(339/596)_a$	34.9% $(66/189)_b$	29.1% $(51/175)_b$	**47.5% (456/960)**
Negative outcomes	23.5% $(140/596)_a$	37.6% $(71/189)_b$	37.1% $(65/175)_b$	**28.8% (276/960)**
No difference	0.8% $(5/596)_a$	2.6% $(5/189)_{a,b}$	4.6% $(8/175)_b$	**1.9% (18/960)**
Described only	12.8% (76/596)	16.9% (32/189)	18.9% (33/175)	**14.7% (141/960)**
Not reported	6.0% (36/596)s	7.9% (15/189)	10.3% (18/175)	**7.2% (69/960)**

Data shown as percentages and raw numbers in brackets. Significant differences ($p < 0.05$) existing between funding categories within rows are indicated by differing subscript letters. (No subscript letters in a row signify no significant differences. The presence of a subscript letter (e.g. 'a' in a cell indicates that it is significantly different from a cell marked with a different letter (e.g. 'b'). If a cell has two subscript letters (e.g. 'a,b') then it is different from cells individually marked with each letter)

remaining 10 (7.9%) having a high risk of bias. Around half of the trials (65/126, 51.6%) were at low risk of bias for incomplete outcome reporting. There was a high risk of bias for incomplete outcome reporting in 19 out of the 126 trials (15.1%) due to missing data, or lack of analysis of the full population of animals randomised in the trial. Twenty-nine of the 126 trials (23.0%) were judged to be at a high risk of bias for selective outcome reporting, only 10/126 (7.9%) were at an unclear risk of bias, and the remaining 87 (69.0%) were assessed as being at a low risk of bias (Fig. 2 and Table 4).

The results of comparing the quality criteria across the trials in different funding are shown in Table 4. The highest percentage of unclear risk for sequence generation was in the pharmaceutical group where 67 out of 86 trials (77.9%) were judged to be at an unclear risk of bias with a lower proportion in the non-pharmaceutical group (12/19, 63.2%) and 3/21 (61.9%) in the no funding declared group (13/21, 61.9%). The pharmaceutical group also had a higher proportion of unclear risk for incomplete outcome reporting in comparison to the other two funding groups (P = 36/86, 41.9%, NP = 3/19, 15.8%, NF = 3/21, 14.3%) and a correspondingly lower proportion of trials in the low risk category for this criteria. The high risk for selective outcome reporting was seen across all the funding categories (P = 18/86, 20.9%; NP = 5/19, 26.3%; NF = 6/21, 28.6%), however the pharmaceutical group had the largest proportion of studies in the low risk category for this criteria compared to the other groups (P = 64/86, 74.4%, NP = 11/19, 57.9%, NF = 12/21, 57.1%). Similar distributions of risk for blinding and allocation concealment were seen across the funding groups (Table 4).

Discussion

This study found a significantly higher proportion of positive outcomes reported in RCTs with pharmaceutical funding (56.9%) or involvement compared to those with declared non-pharmaceutical funding (34.9%) or with no funding source stated (29.1%) within the sample of literature studied. There was a correspondingly lower proportion of negative outcomes reported in trials within the pharmaceutical funding group (23.5%) compared to the other two groups (37.6% and 37.1%). When assessing the trials for risk of bias across the five main categories using the Cochrane risk of bias tool, a large proportion were at an 'unclear' risk indicating significant reporting deficiencies. A high risk of bias was most predominantly seen for selective outcome reporting (reporting bias), and more moderately for incomplete outcome data (attrition bias) and blinding (detection bias). Proportions of trials at high, low or unclear risk of bias for the different quality criteria were largely similar across funding categories.

The sponsorship bias detected in this study is in accordance with many reports in the medical literature where an association between funding source and positive results has been demonstrated, most notably in a Cochrane Review of drug and medical devices [8]. There are many reasons why such a bias may be present in the published literature including differences in the methodological quality of trials; inherent biases in trial conduct to favour a treatment; a genuinely greater likelihood that pharmaceutical companies would be testing pharmaceutical agents that are likely to perform well; and inadequacies in trial reporting which favour a treatment. Additionally, publication bias may play a role through researchers within different environments potentially being more or

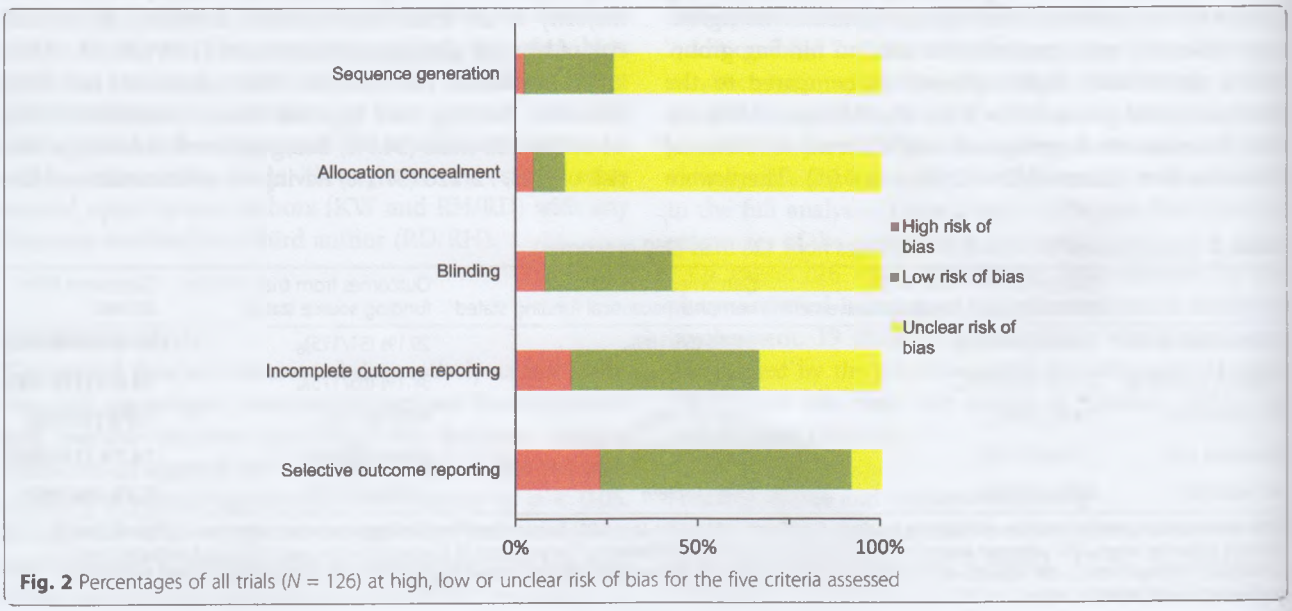

Fig. 2 Percentages of all trials (N = 126) at high, low or unclear risk of bias for the five criteria assessed

Table 4 Risk of bias for trials within different funding categories and overall

	Risk of bias	Pharmaceutical funding/ involvement (86 trials)	Non pharmaceutical funding declared (19 trials)	No funding source declared (21 trials)	All trials (126 trials)
Sequence generation	High	3 (3.5%)	0 (0%)	0 (0%)	**3 (2.4%)**
	Low	16 (18.6%)	7 (36.8%)	8 (38.1%)	**31 (24.6%)**
	Unclear	67 (77.9%)	12 (63.2%)	13 (61.9%)	**92 (73.0%)**
Allocation concealment	High	5 (5.8%)	1 (5.3%)	0 (0%)	**6 (4.8%)**
	Low	5 (5.8%)	3 (15.8%)	3 (14.3%)	**11 (8.7%)**
	Unclear	76 (88.4%)	15 (78.9%)	18 (85.7%)	**109 (86.5%)**
Blinding	High	5 (5.8%)	3 (15.8%)	2 (9.5%)	**10 (7.9%)**
	Low	29 (33.7%)	7 (36.8%)	8 (38.1%)	**44 (34.9%)**
	Unclear	52 (60.5%)	9 (47.4%)	11 (52.4%)	**72 (57.1%)**
Incomplete outcome reporting	High	14 (16.3%)	1 (5.3%)	4 (19.0%)	**19 (15.1%)**
	Low	36 (41.9%)	15 (78.9%)	14 (66.7%)	**65 (51.6%)**
	Unclear	36 (41.9%)	3 (15.8%)	3 (14.3%)	**42 (33.3%)**
Selective outcome reporting	High	18 (20.9%)	5 (26.3%)	6 (28.6%)	**29 (23.0%)**
	Low	64 (74.4%)	11 (57.9%)	12 (57.1%)	**87 (69.0%)**
	Unclear	4 (4.7%)	3 (15.8%)	3 (14.3%)	**10 (7.9%)**

Data expressed as as raw numbers and percentages of total trials

less likely to publish trials demonstrating a positive effect compared to trials showing a 'negative' result. Further studies are required to examine this finding and its potential causative factors in more detail, in particular whether there are correlations between quality criteria and funding source, something which this study did not investigate.

There are a variety of methods that could have been utilised for the current study. For example, in medical literature reviewing the presence of sponsorship bias, it is common to report one overall conclusion for a paper (i.e. overall the paper has a positive/negative/not significantly different outcome) determined either by the reviewers, based on the assertions of the authors or on the statistical analysis of one primary outcome of the study [4, 7, 12]. The method we have used, whereby we have extracted each outcome and its result, is more achievable in the veterinary literature, as primary outcomes are often unspecified [10, 13], but different results would potentially be obtained using a different approach. Of note in this study is the potential for differences between species, and potential clustering of some types of trials, e.g. anthelmintic efficacy trials, to have skewed the data; these limitations will be discussed in more detail below. To date, we have found no other publications examining the association of funding source with positive outcome reporting in the veterinary literature with which to compare our results. The group of trials with no funding source stated are particularly difficult to assess in this study as no assumptions can be made as to which of the two other groups they would most appropriately belong to. Within the results, they appear to be most like the non-pharmaceutical group of trials in their characteristics, but this in itself highlights a continuing problem of poor reporting of clinical trials (20% of trials in this study did not report a funding source).

Selective outcome reporting, for example not reporting, or incompletely reporting, results for pre-specified outcomes, or reporting outcomes that were not pre-specified, can introduce reporting bias into a study and influence the overall results [2, 3]. A striking feature of our data was the high proportion of outcomes that were described only (18.9%) or were mentioned in the materials and methods then not reported in the results (10.3%). This could partly be due to manuscripts not detailing clearly which of the parameters being measured were intended to be outcomes used to assess efficacy, leading us to misclassify the information, highlighting again the issue of poor reporting. A previous study reporting quality criteria and outcome data from a sample of dog and cat trials also reported a high percentage of outcomes with no formal statistical analysis (31%) and a lower percentage not reported at all (3.1%) [10]. The proportions of outcomes in these two categories contribute to the overall high risk of reporting bias (selective outcome reporting) found in this study. Research has shown that outcomes that are not reported, or incompletely reported are more likely to be statistically insignificant [14, 15]. This highlights the need for pre-specified primary and secondary outcomes to be explicitly stated in the methods and adhered to when reporting results. One approach which should help to combat this problem is for all clinical trial protocols to be registered in advance, so a comparison can be made with the final report; this approach is being championed by the AllTrials campaign in human medicine. AllTrials aims to ensure that all clinical trials are registered before they commence and

that all are fully reported [16] (www.alltrials.net). A similar initiative is currently underway for veterinary clinical trials [17]; these schemes should also help to combat publication bias. Publication bias, meaning negative studies are less likely to be published than positive ones, is a problem that has been identified across scientific publishing generally and which can lead to over estimates of treatment effects [3, 15]. The potential impact of publication bias on our study results would depend on who was funding any unpublished trials.

The high proportions of 'unclear' risk of bias for the five quality criteria assessed in this study indicate a significant issue with poor reporting, a feature which has also been described in previous quality assessments of veterinary clinical trial literature [9, 10, 13]. This does not necessarily equate to poor methodological trial conduct, but a lack of complete reporting means that the methodology cannot be adequately assessed [18, 19]. This study did not set out to assess the impact of risk of bias on levels of positive outcome reporting. However, it has previously been shown in both veterinary and medical literature that incomplete or inadequate reporting of certain quality criteria (e.g. method of randomisation) is linked to an exaggeration of treatment efficacy [10, 13, 20, 21].

The CONSORT reporting guideline was developed in order to improve the reporting of RCTs, making it easier to ascertain what was done, identify possible sources of bias, and evaluate the reliability of a study [22, 23]. In general, the adoption of the CONSORT checklist has improved the reporting of RCTs in the medical literature, but there are still reporting deficits [24, 25]. In veterinary medicine the REFLECT statement is also available, which is an extension to the CONSORT reporting guideline specifically developed for RCTs involving livestock [26, 27]. Strict adherence to such reporting guidelines [28] should have reduced all the 'unclear' assessments of bias made in this study and would have allowed us to identify the funding source of all the trials. Most importantly, this would allow more reliable assessments of treatment efficacies to be made, meaning more effective translation of evidence into clinical practice. A recent survey assessing the awareness of reporting guidelines amongst veterinary editors reported that 35.1% of journal editors said reporting guidelines were referred to in their instructions to authors [29]. An improvement in the endorsement of reporting guidelines by journals could help to improve the reporting quality of the veterinary clinical trial literature as it has done for medicine.

A significant limitation of this study is that there were a relatively small number of trials included in the analysis, and due to the large proportion of pharmaceutical trials in the sample (68%), the groups for comparison were unbalanced and the non-pharmaceutical group small. Another, larger study would be extremely beneficial in assessing the presence of sponsorship bias in the veterinary clinical trials literature. In particular, an exploration of potential differences between species, or between companion animal versus production animals, warrants further investigation with larger sample sizes (no significant differences were found in the current study, see Table 2). Results of this type of study can be very dependent on the methods, including what types of studies are included (e.g. we have only included pharmaceutical interventions), which outcome classifications are used, the way in which outcomes are extracted (e.g. we did not include results for outcomes which were not mentioned in the materials and methods) and how funding categories are divided, meaning results across studies could be very different. Another limitation of this study is that the authors were not blinded to any manuscript details during data extraction potentially leading to biased interpretation. The lack of inclusion of efficacy studies where multiple doses of the test treatment were used is another significant limitation of the study. On balance it was felt that inclusion of these could potentially skew the results due to multiple entries for the trial by including each dose, or selecting only one of the doses. The inclusion of multiple trials within one publication may also skew results, as the methods, and therefore assessment of quality, tend to be identical for all the included trials. As most multiple trial papers were in the pharmaceutical category, this could potentially lead to clustering of information. Of particular influence in this study were RCTs assessing anthelmintic agents as these often contained multiple similar trials with an overwhelming proportion of positive outcomes. As they fulfilled our initial inclusion criteria they remained in our sample but their impact on the overall results may be substantial. The subjective assignment of a single outcome result for an outcome which was assessed at multiple time points is another limitation which was necessary for practicality. Limits to the initial sample size were needed due to cost and time constraints; a single calendar year search in PubMed was chosen to give a representative, recent sample of trials, rather than selecting certain journals to search. Using PubMed also allowed us to search by publication type. Not including studies unavailable in English was a necessary cost and time limitation but only one paper was excluded on this basis so this is unlikely to have affected the study outcomes.

Conclusions

This study found a positive association between pharmaceutical funding or involvement and increased positive outcome reporting. Consistently poor reporting of trials, including non-identification of funding source was identified, which hinders the assessment and use of the limited evidence available to the profession.

Additional files

Additional file 1: Cochrane (http://www.cochrane.org/glossary/) definitions of types of bias. Written descriptions of the definitions of the Cochrane types of bias.

Additional file 2: Table S1. Reasons for exclusions of RCTs involving pharmaceutical agents from analysis. Table containing numbers of trials excluded for each reason organised in species groups. (DOCX 14 kb)

Additional file 3: References for all papers included in the analysis within this study (single dose efficacy studies of pharmaceutical interventions in cats, dogs, horses, cattle or sheep published in 2011). List of references in word.

Abbreviations

CONSORT: Consolidated Standards for Reporting Trials; NF: Group of trials for which no funding source was stated; NP: Group of trials for which non-pharmaceutical funding was stated; P: Group of trials for which pharmaceutical funding/involvement was stated; RCT: Randomised controlled trial

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Authors' contributions

All authors were involved in the design of the research project. DG, KW and RH designed the searching strategies. KW, RH and RD extracted and analysed the data. All authors were involved in interpreting the analysed data. KW wrote the draft manuscript. All authors contributed to editing the manuscript. All authors read and approved the final manuscript.

Consent for publication

Not applicable.

Competing interests

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Medicinal plants – prophylactic and therapeutic options for gastrointestinal and respiratory diseases in calves and piglets?

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Abstract

Background: Gastrointestinal and respiratory diseases in calves and piglets lead to significant economic losses in livestock husbandry. A high morbidity has been reported for diarrhea (calves ≤ 35 %; piglets ≤ 50 %) and for respiratory diseases (calves ≤ 80 %; piglets ≤ 40 %). Despite a highly diverse etiology and pathophysiology of these diseases, treatment with antimicrobials is often the first-line therapy. Multi-antimicrobial resistance in pathogens results in international accordance to strengthen the research in novel treatment options. Medicinal plants bear a potential as alternative or additional treatment. Based on the versatile effects of their plant specific multi-component-compositions, medicinal plants can potentially act as 'multi-target drugs'. Regarding the plurality of medicinal plants, the aim of this systematic review was to identify potential medicinal plant species for prevention and treatment of gastrointestinal and respiratory diseases and for modulation of the immune system and inflammation in calves and piglets.

Results: Based on nine initial sources including standard textbooks and European ethnoveterinary studies, a total of 223 medicinal plant species related to the treatment of gastrointestinal and respiratory diseases was identified. A defined search strategy was established using the PRISMA statement to evaluate 30 medicinal plant species starting from 20'000 peer-reviewed articles published in the last 20 years (1994–2014). This strategy led to 418 references (257 *in vitro*, 84 *in vivo* and 77 clinical trials, thereof 48 clinical trials in veterinary medicine) to evaluate effects of medicinal plants and their efficacy in detail. The findings indicate that the most promising candidates for gastrointestinal diseases are *Allium sativum* L., *Mentha x piperita* L. and *Salvia officinalis* L.; for diseases of the respiratory tract *Echinacea purpurea* (L.) MOENCH, *Thymus vulgaris* L. and *Althea officinalis* L. were found most promising, and *Echinacea purpurea* (L.) MOENCH, *Camellia sinensis* (L.) KUNTZE, *Glycyrrhiza glabra* L. and *Origanum vulgare* L. were identified as best candidates for modulation of the immune system and inflammation.

Conclusions: Several medicinal plants bear a potential for novel treatment strategies for young livestock. There is a need for further research focused on gastrointestinal and respiratory diseases in calves and piglets, and the findings of this review provide a basis on plant selection for future studies.

Keywords: Medicinal plants, Calves, Piglets, Gastrointestinal diseases, Respiratory diseases

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Background

A high standard of animal health and welfare is striven in modern livestock husbandries. Health in early life represents a precondition for a superior constitution and results in a high productivity later in life. The mammalian immune system is still immature in the first weeks of life and, in combination with an inappropriate colostral supply, contact to pathogens often results in high morbidity and mortality in young farm animals. Inadequate management including long distance transports, fasting, commingling of individuals from different sources, abrupt changes in diet or incorrect diet, overcrowding of pens, improper climate and suboptimal hygiene are crucially involved in infectious diseases [1–6]. In calves and piglets, the first contact sites for pathogens are the epithelia of the gastrointestinal and respiratory tract. Table 1 shows four of the most important infectious disease complexes in calves and piglets leading to a decreased animal performance and welfare and therefore high economic losses.

In calves and piglets, a variety of pathogens can cause gastrointestinal diseases. Neonatal calf diarrhea represents the most frequent cause of calf losses [2, 7–9] with a mortality of around 55 % in the U.S.A. and in Korea [10] and a morbidity ranging from 12 % in the U.S.A., 23 % in Canada up to 53 % in The Netherlands [1, 7, 11]. Insufficient colostral supply and failure of feeding or improper diet are triggers for diarrhea in calves [12–14]. In suckling and postweaning piglets an infection with enterotoxigenic *Escherichia coli* strains has been reported to lead to high economic losses as a result of a constant high morbidity and mortality [15]. Verocytotoxin-producing *Escherichia coli* infections can lead to more seldom but severe edema disease in weaned pigs [16–18]. The prevalence of postweaning diarrhea has been reported to be 35 % in France [19], the morbidity was stated to exceed 50 % in Finland [20] and the mortality can be as high as 25 % without therapy [17]. The incidence of neonatal diarrhea in piglets depends on concentration of antibodies in sow's colostrum. While piglets are protected by the antibodies in sow's milk, the predisposition for postweaning diarrhea increases with weaning. Additional factors to the immunological gap, including abrupt changes in diet, an increase in stomach pH, and changes in the enzymatic and cellular configuration of the intestine lead to dysbiosis [6, 17, 21].

Respiratory diseases in calves and piglets have been assessed as one of the most serious diseases with regard to financial losses because of decreased weight gain, costs for veterinary interventions and higher condemnation at slaughter [22]. In fattening calves bovine respiratory disease is a considerable challenge with a morbidity ranging from 49 % in Switzerland to 80 % in the U.S.A. [23, 24]. There is a disposition of the bovine respiratory tract to respiratory diseases. Improper microclimate, noxious gases and distress through transportation are predisposing factors additionally [25, 26]. Respiratory diseases are also of high importance in pigs. The morbidity rates differ between countries; a morbidity of 10 and 40 % have been reported for Denmark and the U.S.A. respectively [27, 28]. Mortality rates up to 15 % [29] have been reported and attributed with the porcine respiratory disease complex. The interaction of various pathogens as well as housing conditions, management and genetic factors, were reported to cause bronchopneumonia [27, 29].

Antibiotic therapy is often the first-line therapy of diseases of the gastrointestinal and respiratory tract in calves and piglets. A previously published study showed that fattening calves receive antibiotics for 30 days on average. Moreover, calves are frequently treated with reserve antibiotics such as fluorchinolones and cephalosporines of the 3. and 4. generation [30]. In pig production, routine pro- and metaphylactic administration of antimicrobial agents is a widely-used practice in herds suffering from neonatal diarrhea, postweaning diarrhea or edema disease irrespective of increasing ineffectiveness in consequence of bacterial resistance [6]. More than 60 % of the antibiotics used in porcine husbandry are administered by oral group treatment [31]. Data on antimicrobial resistance monitoring indicated that 59 % of porcine *Escherichia coli* strains from fecal samples showed resistance to at least one antibiotic and 12 % to more than four of the antibiotics that were investigated [32]. With regard to increasing antimicrobial resistance worldwide, the prevailing issue of reducing antibiotics in food producing animals is seeking for novel options to prevent and cure most common and costly diseases. Improved biosecurity and housing conditions, new feeding regimes, vaccinations and the use of disease-resistant breeds are important provisions.

The diverse etiopathology and symptomatology of diseases in young stock is a challenge and demands a multitargeted therapy. In contrast to chemically synthesized mono-target drugs, multi-target drug characteristics are typical for medicinal plants based on their multicomponent composition, which can lead to pleiotropic, synergistic or additive effects in the organism [33, 34]. The broad spectrum of natural products from plants represents a prevailing and widely unemployed potential especially for medication of herbivore and omnivore livestock [35]. Medicinal plants have been used worldwide for prevention and treatment of diseases in human and animals for centuries. Ethnoveterinary research and the underlying documents describing traditional and recent use of medicinal plants [36–38] could be exploited as alternative or as supportive tools to reduce the use of antibiotics in livestock. Additionally, some medicinal plants are known to modulate the immune system and inflammation and could be used as a prophylaxis for infectious diseases.

Human clinical studies, experimental *in vivo*, *ex vivo* and *in vitro* studies on medicinal plants are available,

Table 1 Challenging infectious diseases in calves and piglets: pathogens, pathophysiology, resulting demands for prophylaxis and therapy

Disease complex	Pathogens			Pathophysiology/pathogenesis	Demands for prophylaxis and therapy
	Bacteria	Viruses	Parasites		
Calves					
Neonatal Calf Diarrhea[1]	*Escherichia coli*	Bovine Coronavirus	*Cryptosporidium parvum*	secretory/malabsorptive/ maldigestive diarrhea	antimicrobial
		Rotavirus		dehydration	antiviral
				hypovolemic shock	antidiarrheal
				decrease in temperature	antiadhesive
				d-lactat acidosis	astringent
				septicaemia	spasmolytic
				neurological symptoms	analgesic
				apathy	anti-inflammatory
				recumbency	orexigenic
				reluctance to drink	prebiotic
				fever	immunostimulant
Bovine Respiratory Disease[2]	*Mannheimia haemolytica*	Infectious bovine rhinotracheitis virus		catharral/interstitial/fibrinous bronchopneumonia	antimicrobial
	Pasteurella multocida	Parainfluenza type 3 virus		fever	antiviral
	Histophilus somni	Bovine respiratory syncytial virus		increased respiratory rate	analgesic
	Mycoplasma bovis	Bovine viral diarrhea virus		dyspnoea	anti-inflammatory
				inappetence	immunostimulant
				nasal discharge	mucolytic
				coughing	secretolytic
				apathy	antitussive
				runting	
Piglets					
Neonatal Diarrhea	*Escherichia coli*	Rotavirus	*Cryptosporidium spp.*	secretory/malabsorbtive/ maldigestive diarrhea	antimicrobial
Postweaning Diarrhea[3]	*Clostridium perfringens*	Coronavirus	*Isospora suis*	enteritis	antiviral
	Lawsonia intracellularis	Porcine Circovirus type 2	*Trichuris suis*	colitis	antidiarrheal
	Brachyspira spp.		*Oesophagostomum dentatum*	dehydration	antiadhesive
	Salmonella spp.			acidosis	astringent
	(Yersinia spp.)			septicaemia	spasmolytic
				neurological symptoms	analgesic
				apathy	anti-inflammatory
				reduced growth rates	orexigenic
					prebiotic
					immunostimulant
Porcine respiratory disease complex[4]	*Pasteurella multocida*	Porcine reproductive and respiratory syndrome virus		suppurative/fibrinous/interstitial bronchopneumonia	antimicrobial
	Mycoplasma hyopneumoniae	Swine influenza virus		coughing	antiviral
		Porcine circovirus type 2		nasal discharge	analgesic

Table 1 Challenging infectious diseases in calves and piglets: pathogens, pathophysiology, resulting demands for prophylaxis and therapy *(Continued)*

Actinobacillus pleuropneumoniae		
(Streptococcus suis)	increased respiratory rate	anti-inflammatory
(Haemophilus parasuis)	fever	immunostimulant
	reduced growth rates	mucolytic
	runting	secretolytic
		antitussive

[1][10–13] [2][3–5, 23, 25] [3][6, 15–18] [4][22, 27, 29]

but there is a lack of comprehensive research for veterinary medicine, especially in young farm animals. Therefore, the purpose of the underlying work is to gain information about potential efficacy of medicinal plants in human and veterinary medicine including *in vitro*, *in vivo* and clinical studies.

The aim of this systematic literature review is to identify medicinal plant species or their extracts that are promising candidates for use in diseases of the gastrointestinal and respiratory tract and for stimulation of the immune system and prevention or therapy of inflammation in calves and piglets. Candidate plants should bear a reliable potential for effective treatment and prevention of these diseases. The information obtained can build a basis for state-of-the-art experimental trials and clinical studies with medicinal plants of interest for the treatment of gastrointestinal and respiratory diseases and for the modulation of the immune system and inflammatory processes in calves and piglets.

Methods

The design of the systematic review was 'a priori' individually developed according to the recommendations of the PRISMA statement [39, 40] and AMSTAR measurement tool [41]. The research question was designed following the PICOS scheme [39]: the *population* are calves and piglets in livestock farming, the *intervention* is a treatment with medicinal plants, the *comparator* is no treatment, a placebo or standard treatment, the *outcome* is the effect of the plant, the *study design* included *in vitro*, *ex vivo*, *in vivo* and clinical trials. The detailed protocol of the systematic review is provided in the Additional file 1.

Selection of plant species

To choose eligible plant species, different *initial sources* were screened in respect to plant species frequently recommended for the treatment of gastrointestinal diseases, particularly unspecified or infectious diarrhea and gastrointestinal spasms (QA) and respiratory diseases (QR) as well as those plants that have been reported to modulate the immune system and affect inflammation in infectious diseases (QL). Regarding the intended use of medicinal plants in Western livestock husbandry, potential plant species should be economically available or easy to cultivate in Europe. The *initial sources* included standard literature, based on traditional empiric knowledge and historical literature of veterinary [42–45], and human phytotherapy [46], peer-reviewed publications of European [47] and in particular Swiss ethnoveterinary medicine [36, 38] and a report of the European Food Safety Authority (EFSA) [48] focusing on the use of plants as feed additives in animal production. All plant species of these sources connected to one or more of the indications were recorded including the used part(s) of plant, the route of administration, the dosage, the contraindications and adverse effects (Additional file 1). Based on the plant species that had been mentioned in at least three different *initial sources* for the same indication, a preliminary selection was established. This selection was sent to three specialists in European veterinary phytotherapy to capitalize their experience. The experts were asked to comment on the preliminary selection of plant species regarding the most common ones being particularly suitable for treatment and prevention of gastrointestinal and respiratory diseases.

Selection of scientific references

Bibliographic search

The chosen plant species were included in the following step. A bibliographic web-based search was conducted based on the recommendations of the PRISMA statement [39, 40] and AMSTAR measurement tool [41]. An introduction in scientific bibliographic literature searches and continuous support was provided by a professional librarian. The bibliographic sources used included PubMed [49] and Web of Science [50]. Both were consulted in the time between 2015-02-16 and 2015-02-19 by one person. The search terms consisted of the Latin name, the common trivial name in English and the pharmaceutical denomination in Latin (e.g. "Foeniculum vulgare" OR "fennel" OR "foeniculi fructus"). In the PubMed keyword search, the results were refined with the *subjects* 'complementary

medicine', 'dietary supplements', 'systematic review', 'toxicology' and 'veterinary science'. In the PubMed search with MeshTerms, only the Latin name of the plant was used and the *subheadings* 'adverse effects', 'analysis', 'drug effects', 'microbiology', 'pharmacology', 'therapeutic use', 'therapy' and 'toxicity'. In the Web of Science database, the search was conducted in the *research domain* 'science technology' and the results were refined with the *research areas* 'pharmacology', 'infectious diseases', 'toxicology', 'veterinary sciences', 'microbiology', 'gastroenterology', 'integrative complementary medicine', 'general internal medicine', 'respiratory system' and 'virology'. Overall, only peer-reviewed articles written in English or German language and published between 1994 and 2014 were considered for further evaluation to ensure contemporary scientific quality and timeliness of the review. The references found were saved in an EndNote X7 data base [51] and the information on each plant was stored in a separate folder in this database. Duplicates were removed for each plant species.

In some studies more than one plant species was investigated (e. g. a screening of plant species against *E. coli* [52]). In other studies, more than one indication was considered and investigated (e. g. spasmolytic effect of *Plantago lanceolata* L. on intestine and trachea [53]). Therefore, the following definition of "reference" was introduced:

> reference = indication per plant species per peer-reviewed publication

Term-list search

A term-list search was conducted within each plant species using the search function in EndNote X7 [51]. Only references containing one of the following predefined keywords occurring in the title or the abstract were included: 'pig*', 'calv*', 'muco*', 'spasmo*', 'anti*' (e.g. antimicrobial, antibacterial, antiviral, antifungal, antioxidant, antinociceptive...), 'wean*', 'intest*', 'gastro*', 'pulmo*', 'broncho*', 'pharma*', 'eff*'(e.g. efficiency, effectivity, effect), 'bioactiv*', 'constitu*'. References containing the terms 'tumor' or 'cancer' were excluded. The check of the excluded references lowered the risk to exclude relevant references.

Refining with inclusion and exclusion criteria

The remaining references were refined using a selective screening of the title. References remained if the content suffices the objective of the review. Therefore, inclusion criteria were pre-defined by two scientists and lead to an inclusion of all references containing investigations of plants in *in vitro*, *ex vivo*, *in vivo* or clinical studies. Besides these categories, the evaluation included the following inclusion criteria: antibacterial effect, enhancement of antibiotics, antiviral effect, antiprotozoal effect, anti-inflammatory effect, analgesic effect, spasmolytic effect, antiadhesive effect, astringent effect, secretolytic or mucolytic effect, antitussive effect, and other effects on the gastrointestinal tract, respiratory tract or immune system, treatment of diarrhea, bacterial or viral infections of the gastrointestinal tract or respiratory tract, bronchopneumonia, common cold, cough as well as ingredients, constituents, components of plants and the detection or extraction of them, toxic activity or adverse effects due to a treatment with plants.

Exclusion criteria were chosen in order to exclude references dealing with other plant species or subspecies than those we focused on, a mixture of different plant species investigated as one single preparation, pathogens affecting only humans, diseases regulated by laws, cultivation or breeding of plants, plant genetics, seeds and fertilizers, regional reservoirs, habitats or demands for growing of plants, plant pathology, plant protection systems or pesticides, ecology, geology, ethology, sociology, the usage of the plant as food, food technology or food-packaging, the use of the plant as a repellent or insecticide, other medical branches, other diseases or apparatuses than mentioned in the inclusion criteria (e.g. dermatology, cardiology, oncology, nephrology, diabetes) as well as other animal classes than mammalians and birds.

Classification

Thereby the references were classified into different categories of trial types. Studies investigating diseases occurring naturally in the investigated animal species or in humans were categorized as 'clinical references'. Trials investigating diseases or the effect of plants in animal models were categorized as '*in vivo* references'. Studies using pathogens, cell layers or *ex vivo* models were categorized as '*in vitro* references'. Studies investigating the pharmacologic characteristics, constituents or the detection of them were categorized as 'pharmacognostic references' and the evaluation of plants summarizing other studies as 'review references'.

In the last step, abstracts of the remaining clinical, *in vivo* and *in vitro* references were studied by one person. During this process, further references were excluded because they did not match the predefined selection criteria.

Assessment of clinical, in vivo and in vitro references

The remaining references were assessed by the following characteristics: used plant species, type of reference (clinical, *in vivo* or *in vitro*), indication of the reference inspired by the ATCvet classification (QA, QR, QL) [54], animal species used, study design, pharmaceutical form of the plant, type of application, concentration tested, dosage or minimal inhibition concentration and, if available, the tested pathogen.

To assess the potential of the selected plant species, a reconciliation of the demands for prophylaxis and therapy of gastrointestinal and respiratory diseases with the

hypothesized and tested effects of the plants was performed. The demands for prophylaxis and therapy were derived from the pathophysiology of the focused diseases (Table 1). According to these data, plant-derived treatment options should act bacteriostatic or bactericidal, synergistically with antibiotics, antiviral, antiprotozoal, anti-inflammatory, analgesic, immunomodulatory, antidiarrheal, antiadhesive, spasmolytic, astringent, expectorant or antitussive (depending on the indication). The conclusion of a trial on the investigated hypothesized effect of the plant species (Additional file 3) was transferred in the following assessment. To compare the potential of the plant species, a scoring system was established. For each significantly proven effect, the plant species one point was given, while for each uncertain effect, zero points were assigned, and for each disproved effect a point was subtracted (for more details see Additional file 1). Clinical studies were given more weight compared to *in vivo* studies followed by *in vitro* studies. Clinical studies were given a weight of three, *in vivo* studies two, and *in vitro* studies one. The weighted average of the sum of points of the clinical, *in vivo*, and *in vitro* scores served as the final score. The scores were used to identify the plant species that are the most efficacious options for related disease complexes.

$$\begin{aligned}\text{Score} = {} & 3 \times (\text{number of proven effects in clinical studies} - \\ & \text{number disproof of effects in clinical studies}) + \\ & 2 \times (\text{number of proven effects in } \textit{in vivo} \text{ studies} - \\ & \text{number ofdisproof of effects in } \textit{in vivo} \text{ studies}) + \\ & 1 \times (\text{number of proven effects in } \textit{in vitro} \text{ studies} - \\ & \text{number of disproof of effects of } \textit{in vitro} \text{ studies})\end{aligned}$$

Results

The procedure of this systematic literature review is visualized in Fig. 1. The screening of ethnoveterinary research and standard phytotherapeutic textbooks (*initial sources*) led to a total of 223 plant species recommended for the treatment and prophylaxis of gastrointestinal (diarrhea and intestinal spasms) and respiratory diseases in human and animals. A number of 134 different plant species were recommended for QA, 121 for QR and 44 for QL (Additional file 2). A preliminary selection of 29 plant species, recommended in at least three different sources for the same indication, was established. Therefrom, 17 plant species were recommended for QA, 15 for QR and 8 for QL. The specialists review led to an addition of one plant species (*Origanum vulgare* L.) to the preliminary list including finally 30 plant species. All of these plant species meet the claims for cost-efficiency or cultivability in Europe.

In the subsequent bibliographic search 20,364 references (after removal of duplicates) were found for the 30 plants species (Table 2). During the term-list search, the amount of relevant references led to a reduction of references with 6,800 remaining references. An ensuing random check of the excluded references confirmed the selected terms. The subsequent screening of titles led to a number of 2,797 eligible references, which were classified into the categories 'clinical references' (243), '*in vivo* references' (428), '*in vitro* references' (1258), 'pharmacognostic references' (704) and 'review references' (164). The terminal screening of the abstracts of all clinical, *in vivo* and *in vitro* references revealed a final number of 418 references (77 clinical, 84 *in vivo*, 258 *in vitro*) (Additional file 3). Due to the fact that more than one reference could be defined from some studies, the systematic literature research led to a number of 378 studies representing the effects and efficacy of 29 plant species in 418 references. For one plant species, *Quercus robur* L., no references were found according to the criteria.

A total of 19,077 references were excluded because they did not match the predefined selection criteria. Predominant reasons for exclusion included that the content of title and abstract did not correspond to the focus of the review (e. g. pathogens were not the pathogens of the focused diseases). Other reasons were missing abstracts (in 212 references) or publications that were not peer-reviewed.

From the 418 remaining references, 48 references based on clinical studies were veterinary origin with 19 swine studies, 5 cattle studies, 17 horse studies, and 4 studies in rabbits. A number of 370 references include studies in humans (29 clinical, 84 *in vivo* and 257 *in vitro* studies). A number of 77 *in vivo* references used laboratory rodents (rats, mice, guinea-pigs) and three studies used cats as an animal model. For gastrointestinal indications (QA), 198 references were found, 57 references were related to respiratory diseases (QR), and 163 references aimed at the modulation of the immune system and inflammation processes (QL). Most references coping with the inclusion criteria were found for *Echinacea purpurea* L. MOENCH. (48 references), *Origanum vulgare* L. (36 references) *Thymus vulgaris* L. (36 references), *Camellia sinensis* (L.) KUNTZE (32 references), and *Allium sativum* L. (31 references). The required effects of a treatment and the proven effects of the plant species as mono-substances for each indication are shown in Tables 3, 4 and 5. In Table 6, the most promising plant species of the peer-reviewed references according to the scoring system for each indication (QA, QR and QL), as well as the most frequently recommended plant species of the traditional references (Additional file 2) are shown. According to the scoring system, the two most promising plant species are *Echinacea purpurea* (L.) MOENCH (for QR and QL) and *Allium sativum* L. (for QA).

Discussion

There is a large amount of evidence-based knowledge about medicinal plants, represented by 20,364 studies focusing on 30 medicinal plant species from the last

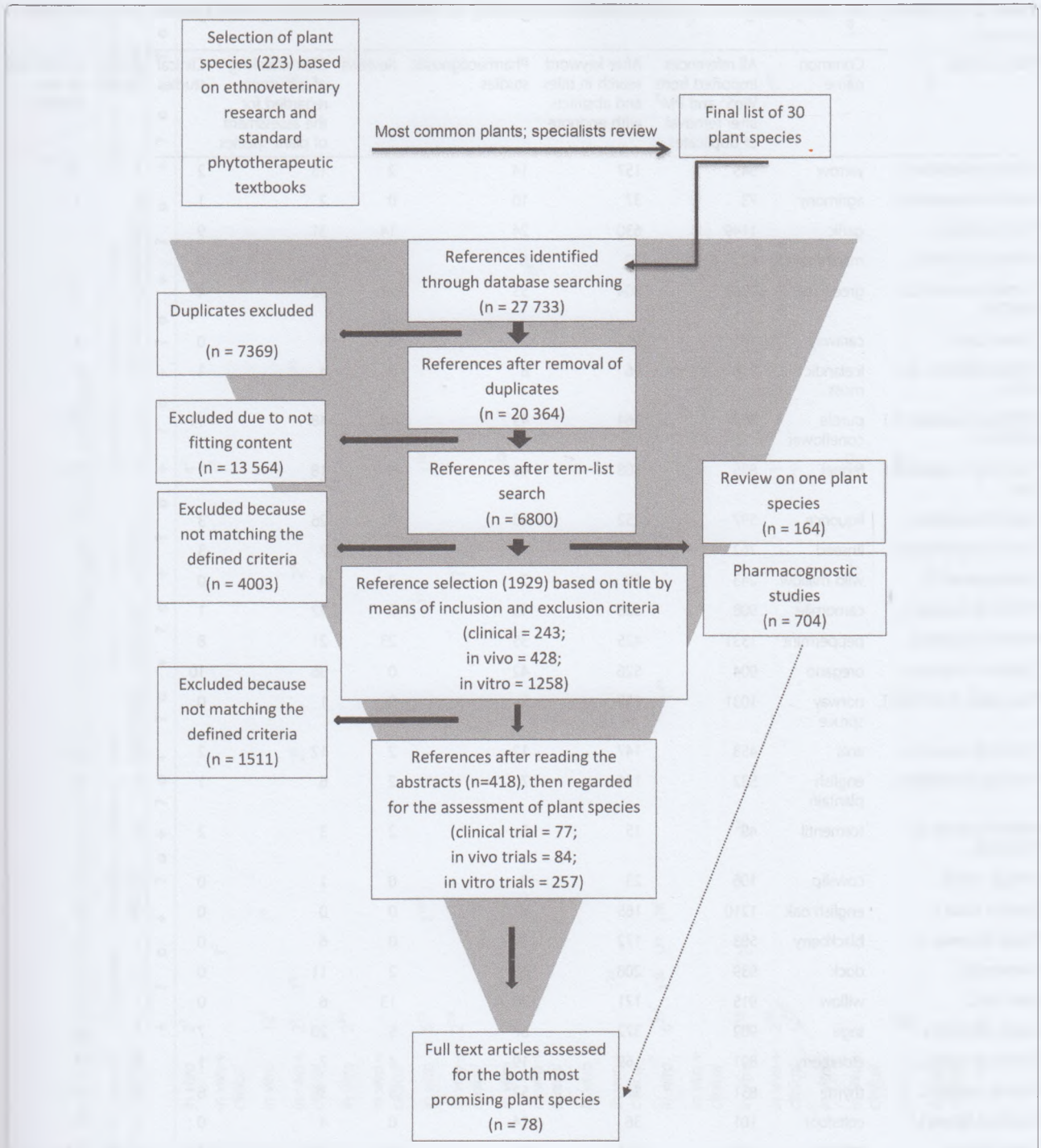

Fig. 1 Process of the systematic literature review

20 years considering peer-reviewed publications in English or German language. The emergence of multi-drug resistance in human and animal pathogens results in international accordance to strengthen the research in novel treatment options. Medicinal plants and their extracts might be an option to prevent and cure livestock diseases.

Evaluation of the search strategy

This systematic review was designed and performed according to the guidelines of the PRISMA statement and AMSTAR measurement tool [39–41]. Due to the fact that we searched for available data on a largely underrepresented topic in the last decades, only a small number of

Table 2 Quantifying and categorizing of scientific publications regarding 30 medicinal plants during bibliographic literature research process

Plant species	Common name	All references imported from WoS[1] and PM[2] after removal of duplicates	After keyword search in titles and abstracts with endnote	Pharmacognostic studies	Reviews	After checking of relevance, regarded for the assessment of plant species	Clinical studies	*In vivo* studies	*In vitro* and *ex vivo* studies
Achillea millefolium L.	yarrow	345	157	14	2	15	2	3	10
Agrimonia eupatoria L.	agrimony	73	37	10	0	2	1	0	1
Allium sativum L.	garlic	1149	630	24	14	31	9	5	17
Althaea officinalis L.	marshmellow	62	29	6	1	6	0	4	2
Camellia sinensis (L.) KUNTZE	green tea	2052	804	53	21	32	4	6	22
Carum carvi L.	caraway	191	95	28	6	5	0	1	4
Cetraria islandica (L.) ACH.	Icelandic moss	118	46	8	0	4	1	1	2
Echinacea purpurea (L.) MOENCH	purple coneflower	869	364	45	18	48	14	8	26
Foeniculum vulgare (L.) MILL.	fennel	825	308	46	6	18	1	4	13
Glycyrrhiza glabra L.	liquorice	597	252	43	8	26	3	7	16
Linum usitatissimum L.	linseed	762	227	12	5	7	3	3	1
Malva sylvestris L.	wild mallow	243	50	2	2	4	0	3	1
Matricaria recutita L.	camomile	908	305	43	7	22	1	7	14
Mentha x piperita L.	peppermint	1331	425	35	23	21	8	0	13
Origanum vulgare L.	oregano	904	526	42	0	36	10	4	22
Picea abies (L.) H.KARST.	norway spruce	1031	153	0	0	1	0	0	1
Pimpinella anisum L.	anis	453	147	12	2	12	2	0	10
Plantago lanceolata L.	english plantain	532	136	7	2	6	1	0	5
Potentilla erecta (L.) RAEUSCH.	tormentil	49	15	3	2	3	2	0	1
Primula veris L.	cowslip	106	23	2	0	1	0	0	1
Quercus robur L.	english oak	1210	165	7	0	0	0	0	0
Rubus fruticosus L.	blackberry	583	172	34	0	6	0	1	5
Rumex ssp. L.	dock	939	208	15	2	11	0	4	7
Salix ssp. L.	willow	915	171	20	13	6	0	1	5
Salvia officinalis L.	sage	902	372	67	5	20	7	7	6
Sambucus nigra L.	elderberry	891	169	19	4	7	1	2	4
Thymus vulgaris L.	thyme	831	372	52	3	36	6	2	28
Tussilago farfara L.	coltsfoot	101	36	14	0	4	0	1	3
Urtica dioica L.	stinging nettle	760	251	16	10	20	1	6	13
Vaccinium myrtillus L.	blueberry	632	155	25	7	8	0	4	4
sum[a]		20364	6800	704	164	418	77	84	257

[a]due to the definition of reference (trial x plant species x indication) the sum may contain some trials more than one time; [1]WoS = Web of Science [50]; [2]PM = PubMed [49]

veterinary clinical data is currently available. Therefore, the search strategy was adapted to gain as much plant specific information as possible and to cope with the complex research question. Human clinical studies, experimental *in vivo* studies with laboratory animals as well as *ex vivo* and *in vitro* studies were included as well. To avoid the

Table 3 Assessment[a] of medicinal plants based on peer-reviewed references[b] of the last 20 years aiming gastrointestinal indications[c]

Plant species	Number of references	Type of reference	Antibacterial			Synergism with AB			Antiviral			Anti protozoal			Anti adhesive			Anti diarrheal			Spasmolytic			Immuno stimulant			Antiin flammatory			Analgesic			Hypothesis proved		
			+	?	o	+	?	o	+	?	o	+	?	o	+	?	o	+	?	o	+	?	o	+	?	o	+	?	o	+	?	o	+	?	o
Achillea millefolium L.	6	*in vitro*	2[1]																		4[2]												
	2	*in vivo + clinical*			1[3]																1[4]												
Allium sativum L.	12	*in vitro*	12[5]			1[6]																											
	9	*in vivo + clinical*	2[7]	1[8]								2[9]						2[10]						1[11]			1[12]						2[13]		
Althaea officinalis L.	2	*in vitro*	2[14]																														
	0	*in vivo + clinical*																															
Camellia sinensis (L.) KUNTZE	8	*in vitro*	6[15]			1[16]									1[17]						1[18]												
	3	*in vivo + clinical*	2[19]															2[20]															
Carum carvi L.	4	*in vitro*	3[21]																		1[22]												
	1	*in vivo + clinical*																									1[23]						
Echinacea purpurea (L.) MOENCH	0	*In vitro*																															
	1	*in vivo + clinical*		1[24]																													
Foeniculum vulgare (L.) MILL.	10	*in vitro*	6[25]	1[26]	1[27]	1[28]							1[29]														1[30]						
	2	*in vivo + clinical*																			1[31]												1[32]
Glycyrrhiza glabra L.	9	*in vitro*	4[33]		2[34]				1[35]												1[36]						1[37]						
	4	*in vivo + clinical*	2[38]																		1[39]											1[40]		
Linum usitatissimum L.	0	*in vitro*																															
	3	*in vivo + clinical*																1[41]			1[42]											1[43]		1[44]
Matricaria recutita L.	9	*in vitro*	5[45]																		4[46]												
	3	*in vivo + clinical*																2[47]														1[48]		

Table 3 Assessment[a] of medicinal plants based on peer-reviewed references[b] of the last 20 years aiming gastrointestinal indications[c] *(Continued)*

Plant species	Number of references	Type of reference	Antibacterial			Synergism with AB			Antiviral			Anti protozoal			Anti adhesive			Anti diarrheal			Spasmolytic			Immuno stimulant			Antiin flammatory			Analgesic			Hypothesis proved		
			+	?	o	+	?	o	+	?	o	+	?	o	+	?	o	+	?	o	+	?	o	+	?	o	+	?	o	+	?	o	+	?	o
Mentha x piperita L.	13	*in vitro*	8[49]	1[50]		1[51]															3[52]													
	7	*in vivo* + clinical	1[53]															1[54]			4[55]											1[56]		
Origanum vulgare L.	21	*in vitro*	18[57]	2[58]																							1[59]							
	8	*in vivo* + clinical	1[60]									2[61]						1[62]		1[63]				2[64]			1[65]					1[66]		
Picea abies (L.) H.KARST.	1	*in vitro*	1[67]																															
	0	*in vivo* + clinical																																
Pimpinella anisum L.	7	*in vitro*	3[68]	2[69]		2[70]															1[71]													
	0	*in vivo* + clinical																																
Plantago lanceolata L.	1	*in vitro*																			1[72]													
	1	*in vivo* + clinical			1[73]																													
Potentilla erecta (L.) RAEUSCH.	0	*in vitro*																																
	2	*in vivo* + clinical													1[74]												1[75]							
Rubus fruticosus L.	1	*in vitro*										1[76]																						
	0	*in vivo* + clinical																																
Rumex ssp. L.	4	*in vitro*	4[77]																															
	1	*in vivo* + clinical																1[78]																
Salix ssp. L.	1	*in vitro*																			1[79]													
	0	*in vivo* + clinical																																
Salvia officinalis L.	6	*in vitro*	5[80]	1[81]																														
	5	*in vivo* + clinical	3[82]									2[83]						1[84]	1[85]		1[86]			1[87]										
Sambucus nigra L.	1	*in vitro*	1[88]																															
	1	*in vivo* + clinical																									1[89]							

Table 3 Assessment[a] of medicinal plants based on peer-reviewed references[b] of the last 20 years aiming gastrointestinal indications[c] *(Continued)*

Thymus vulgaris L.	14	*in vitro*	10[60]	1[91]																		3[92]														
	3	*in vivo* + clinical				2[93]																			1[94]											
Plant species	Number of references	Type of reference	Antibacterial			Synergism with AB			Antiviral			Anti protozoal			Anti adhesive			Anti diarrheal			Spasmolytic			Immuno stimulant			Antiin flammatory			Analgesic			Hypothesis proved		
			+	?	o	+	?	o	+	?	o	+	?	o	+	?	o	+	?	o	+	?	o	+	?	o	+	?	o	+	?	o	+	?	o		
Tussilago farfara L.	1	*in vitro*	1[95]																																	
	0	*in vivo* + clinical																																		
Urtica dioica L.	6	*in vitro*	2[96]			2[97]			2[98]																											
	2	*in vivo* + clinical																									2[99]									
Vaccinium myrtillus L.	2	*in vitro*					1[100]					1[101]																								
	2	*in vivo* + clinical																							1[102]		1[103]		1[104]							
Sum of plant species for each effect		*in vitro*	18	6	3	6	0	0	2	0	0	2	0	1	0	0	1	0	0	0	10	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0
		in vivo + clinical	6	2	3	0	0	0	0	0	0	3	0	0	1	0	0	7	2	1	6	0	0	3	0	2	6	1	1	0	0	0	6	0	2	
Sum of assessments		*in vitro*	93	8	5	7	0	0	3	0	0	2	0	0	0	0	1	0	0	0	20	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0
		in vivo + clinical	11	2	4	0	0	0	0	0	0	6	0	0	1	0	0	10	2	1	7	0	0	3	0	3	7	1	1	0	0	0	7	0	2	

[a]Assessment = conclusion of a reference on a hypothesized effect; [b]reference = trial x plant species x indication; [c]particularly unspecified or infectious diarrhea and gastrointestinal spasms + = reference proves evidently the hypothesized effect; ? = reference shows uncertain hypothesized effect; o = reference does not prove evidently the hypothesized effect

[1][147, 148] [2][149–152] [3][153] [4][154] [5][69–76, 155–158] [6][69] [7][63, 66] [8][159] [9][62, 160] [10][63, 64] [11][66] [12][77] [13][161, 162] [14][163, 164] [15][165–170] [16][170] [17][171] [18][172] [19][124, 125] [20][124, 173] [21][174–176] [22][177] [23][178] [24][179] [25][175, 176, 180–183] [26][184] [27][185] [28][181] [29][186] [30][187] [31][188] [32][189] [33][143, 190–192] [34][142, 193] [35][145] [36][194] [37][195] [38][196, 197] [39][141] [40][198] [41][199] [42][199] [43][200] [44][201] [45][202] [175, 185, 203, 204] [46][205–208] [47][209, 210] [48][189] [49][176, 211–216] [50][217] [51][214] [52][88, 205, 218] [53][219] [54][220] [55][86, 87, 221, 222] [56][223] [57][52, 118, 175, 224–237] [58][238, 239] [59][240] [60][241] [61][242, 243] [62][244] [63][245] [64][244, 246] [65][247] [66][248] [67][249] [68][175, 250, 251] [69][252, 253] [70][251, 254] [71][255] [72][53] [73][256] [74][257] [75][258] [76][259] [77][180, 260–262] [78][263] [79][264] [80][175, 211, 233, 237, 265] [81][252] [82][266–268] [83][267, 268] [84][269] [85][270] [86][269] [87][268] [88][271] [89][272] 90[52, 118–120, 175, 176, 226, 250, 252, 273] [91][274] [92][113, 275, 276] [93][277, 278] [94][279] [95][280] [96][191, 281] [97][185, 282] [98][145] [99][283, 284] [100][285] [101][259] [102][286] [103][287] [104][286]

Table 4 Assessment[a] of medicinal plants based on peer-reviewed references[b] of the last 20 years aiming respiratory indications

Plant species	Number of references	Type of reference	Anti-bacterial			Synergism with AB			Antiviral			Spasmolytic			Expectorant			Antitussive			Immuno-stimulant			Anti-inflammatory			Analgesic			Hypothesis proved		
			+	?	o	+	?	o	+	?	o	+	?	o	+	?	o	+	?	o	+	?	o	+	?	o	+	?	o	+	?	o
Achillea millefolium L.	2	*in vitro*										2[1]																				
	1	*in vivo* + clinical										1[2]																				
Agrimonia eupatoria L.	1	*in vitro*							1[3]																							
	0	*in vivo* + clinical																														
Allium sativum L.	1	*in vitro*										1[4]																				
	0	*in vivo* + clinical																														
Althaea officinalis L.	0	*in vitro*																														
	4	*in vivo* + clinical																4[5]														
Camellia sinensis (L.) KUNTZE	0	*in vitro*																														
	2	*in vivo* + clinical	1[6]					1[7]							1[8]																	
Cetraria islandica (L.) ACH.	0	*in vitro*																														
	1	*in vivo* + clinical																						1[9]								
Echinacea purpurea (L.) MOENCH	3	*in vitro*							2[10]															2[11]								
	8	*in vivo* + clinical	1[12]						1[13]		1[14]										2[15]		1[16]							2[17]		2[18]
Foeniculum vulgare (L.) MILL.	2	*in vitro*										2[19]																				
	0	*in vivo* + clinical																														
Glycyrrhiza glabra L.	1	*in vitro*							1[20]																							
	2	*in vivo* + clinical										1[21]						1[22]														
Mentha x piperita L.	1	*in vitro*										1[23]																				
	0	*in vivo* + clinical																														
Pimpinella anisum L.	2	*in vitro*										2[24]																				
	0	*in vivo* + clinical																														
Plantago lanceolata L.	1	*in vitro*										1[25]																				
	0	*in vivo* + clinical																														
Primula veris L.	1	*in vitro*	1[26]																													
	0	*in vivo* + clinical																														
Rubus fruticosus L.	0	*in vitro*																														
	1	*in vivo* + clinical																						1[27]								
Rumex ssp. L.	2	*in vitro*	1[28]						2[29]																							
	0	*in vivo* + clinical																														

Table 4 Assessment[a] of medicinal plants based on peer-reviewed references[b] of the last 20 years aiming respiratory indications *(Continued)*

Salvia officinalis L.	0	*in vitro*																																		
	2	*in vivo* + clinical																																		
Sambucus nigra L.	1	*in vitro*																																		
	1	*in vivo* + clinical																																		
Thymus vulgaris L.	9	*in vitro*																																		
	1	*in vivo* + clinical																																		
Tussilago farfara L.	1	*in vitro*																																		
	1	*in vivo* + clinical																																		
Urtica dioica L.	3	*in vitro*																																		
	1	*in vivo* + clinical																																		
Vaccinium myrtillus L.	1	*in vitro*																																		
	0	*in vivo* + clinical																																		
Sum of plant species for each effect		*in vitro*	5	0	0	0	0	0	6	0	0	7	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0				
		in vivo + clinical	2	0	0	0	0	1	2	0	1	2	0	0	2	0	0	4	0	0	1	0	0	2	0	0	1	0	0	3	0	1				
Sum of assessments		*in vitro*	9	0	0	0	0	0	8	0	0	15	0	0	0	0	0	0	0	0	0	0	1	2	0	0	0	0	0	0	0	0				
		in vivo + clinical	2	0	0	0	0	1	2	0	1	2	0	0	2	0	0	7	0	0	2	0	0	2	0	0	1	0	0	4	0	2				

[a] Assessment = conclusion of a reference on a hypothesized effect; [b] reference = trial x plant species x indication; + = reference proves evidently the hypothesized effect; **?** = reference shows uncertain hypothesized effect; **o** = reference does not prove evidently the hypothesized effect

[1] [288, 289] [2] [290] [3] [291] [4] [292] [5] [293–296] [6] [297] [7] [298] [8] [297] [9] [299] [10] [300, 301] [11] [103, 301] [12] [302] [13] [94] [14] [96] [15] [302, 303] [16] [96] [17] [95, 304] [18] [305, 306] [19] [307, 308] [20] [309] [21] [140] [22] [139] [23] [89] [24] [310, 311] [25] [53] [26] [312] [27] [313] [28] [314] [29] [315] [30] [293] [31] [316] [32] [300] [33] [317] [34] [191, 318, 319] [35] [113–116, 320, 321] [36] [108] [37] [322] [38] [323] [39] [323] [40] [322, 324, 325] [41] [326] [42] [327]

Table 5 Assessment[a] of medicinal plants based on peer-reviewed references[b] of the last 20 years aiming the modulation of the immune system and inflammation

Plant species	Number of references	Type of reference	Immunostimulant			Antiinflammatory			Analgesic			Hypothesis proved		
			+	?	o	+	?	o	+	?	o	+	?	o
Achillea millefolium L.	2	*in vitro*				2[1]								
	2	*in vivo* + clinical			1[2]			1[3]			1[4]			
Agrimonia eupatoria L.	0	*in vitro*												
	1	*in vivo* + clinical				1[5]								
Allium sativum L.	4	*in vitro*	1[6]	1[7]		2[8]								
	5	*in vivo* + clinical	5[9]			1[10]								
Camellia sinensis (L). KUNTZE	14	*in vitro*	3[11]			12[12]								
	5	*in vivo* + clinical	2[13]			2[14]			1[15]					
Cetraria islandica (L.) ACH.	2	*in vitro*	2[16]											
	1	*in vivo* + clinical				1[17]								
Echinacea purpurea (L.) MOENCH	23	*in vitro*	16[18]	1[19]		6[20]								
	13	*in vivo* + clinical	9[21]		2[22]							1[23]		1[24]
Foeniculum vulgare (L.) MILL.	1	*in vitro*				1[25]								
	3	*in vivo* + clinical				1[26]			3[27]					
Glycyrrhiza glabra L.	6	*in vitro*	4[28]			2[29]								
	4	*in vivo* + clinical	3[30]			1[31]			1[32]					
Linum usita-tissimum L.	1	*in vitro*				1[33]								
	3	*in vivo* + clinical		1[34]		2[35]			1[36]					
Malva sylvestris L.	1	*in vitro*				1[37]								
	3	*in vivo* + clinical	1[38]			2[39]			1[40]					
Matricaria recutita L.	5	*in vitro*		1[41]		4[42]								
	5	*in vivo* + clinical	1[43]		1[44]	2[45]			2[46]					
Mentha x piperita L.	0	*in vitro*												
	1	*in vivo* + clinical							1[47]					
Origanum vulgare L.	1	*in vitro*				1[48]								
	6	*in vivo* + clinical	2[49]						3[50]				1[51]	
Pimpinella anisum L.	1	*in vitro*				1[52]								
	2	*in vivo* + clinical	1[53]	1[54]										
Plantago lanceolata L.	3	*in vitro*				3[55]								
	0	*in vivo* + clinical												
Potentilla erecta (L.) RAEUSCH.	1	*in vitro*				1[56]								
	0	*in vivo* + clinical												
Rubus fruticosus L.	4	*in vitro*				4[57]								
	0	*in vivo* + clinical												
Rumex ssp. L.	1	*in vitro*				1[58]								
	3	*in vivo* + clinical				2[59]			2[60]					
Salix ssp. L.	4	*in vitro*				4[61]								
	1	*in vivo* + clinical				1[62]								
Salvia officinalis L.	0	*in vitro*												
	7	*in vivo* + clinical	1[63]	1[64]	1[65]	4[66]			2[67]					
Sambucus nigra L.	2	*in vitro*	1[68]			1[69]								
	1	*in vivo* + clinical			1[70]									

Table 5 Assessment[a] of medicinal plants based on peer-reviewed references[b] of the last 20 years aiming the modulation of the immune system and inflammation *(Continued)*

Thymus vulgaris L.	5	*in vitro*				5[71]								
	4	*in vivo* + clinical		1[72]	1[73]				2[74]					
Tussilago farfara L.	1	*in vitro*				1[75]								
	0	*in vivo* + clinical												
Urtica dioica L.	4	*in vitro*	2[76]			2[77]								
	4	*in vivo* + clinical	3[78]			1[79]			1[80]					
Vaccinium myrtillus L.	1	*In vitro*				1[81]								
	2	*in vivo* + clinical	1[82]			2[83]								
Sum of plant species for each effect		*in vitro*	6	3	0	20	0	0	0	0	0	0	0	0
		in vivo + clinical	9	4	6	13	0	1	12	0	1	1	1	1
Sum of assessments		*in vitro*	29	3	0	56	0	0	0	0	0	0	0	0
		in vivo + clinical	24	4	7	20	0	1	20	0	1	1	1	1

[a]Assessment = conclusion of a reference on a hypothesized effect; [b]reference = trial x plant species x indication; + = reference proves evidently the hypothesized effect; **?** = reference shows uncertain hypothesized effect; **o** = reference does not prove evidently the hypothesized effect
[1][328, 329] [2][330] [3][331] [4][331] [5][332] [6][333] [7][334] [8][78, 80] [9][65, 67, 68, 79, 335] [10][79] [11][133, 336, 337] [12][336, 338–348] [13][134, 349] [14][131, 350] [15][351] [16][352, 353] [17][354] [18][99, 100, 355–368] [19][369] [20][102, 370–374] [21][93, 97, 98, 375–380] [22][381, 382] [23][383] [24][384] [25][385] [26][386] [27][386–388] [28][144, 334, 389, 390] [29][391, 392] [30][138, 380, 393] [31][394] [32][394] [33][395] [34][396] [35][397, 398] [36][397] [37][399] [38][400] [39][401, 402] [40][401] [41][403] [42][404–407] [43][408] [44][409] [45][410, 411] [46][410, 412] [47][413] [48][414] [49][415, 416] [50][417–419] [51][420] [52][421] [53][422] [54][423] [55][392, 424, 425] [56][426] [57][427–430] [58][431] [59][432, 433] [60][432, 434] [61][426, 435–437] [62][438] [63][439] [64][440] [65][441] [66][442–445] [67][442, 443] [68][446] [69][430] [70][447] [71][436, 448–450] [72][451] [73][451] [74][417, 452] [75][453] [76][454, 455] [77][456, 457] [78][138, 458, 459] [79][460] [80][460] [81][344] [82][461] [83][461, 462]

risk of source selection bias, multiple types of sources were used initially: standard textbooks, peer-reviewed publications, a governmental report, and personal communications with experts. The risk of introducing database bias was reduced by using two different and independent databases and by using the Mesh Terms function of PubMed. The selection of the 30 traditionally used plant species may bear a sampling bias. European ethnoveterinary and traditional administrations of medicinal plants were screened to identify promising plant species for the bibliographic search. Due to our strategy, it is likely that frequently studied plant species come up as more promising compared to less frequently studied plants. Additionally, the timeliness of the review excluded studies published before 1994 which may be accepted in science for decades by e.g. the European Scientific Cooperative on Phytotherapy [55]. As a consequence, plants including *Malva sylvestris* L., *Potentilla erecta* (L.) RAEUSCH, *Primula veris* L., *Quercus robur* L. or *Picea abies* (L.) H.KARST. appeared less promising, although they are an integral element of traditional medicine.

Comparison of traditional phytotherapy with up-to-date knowledge

The most promising plant species of the peer-reviewed publications of the last 20 years were compared to the most common traditional administrations of the *initial sources* (Table 6). For the most promising plant species 62, 30 and 27 % are also frequently recommended in the *initial sources* for QA, QR and QL, respectively. The results confirmed the rationale of some traditional administrations of medicinal plants. Cases where the traditional applications were not confirmed by current studies, may be explained by the fact that only studies published between 1994 and 2014 were considered. Nevertheless, for many of these plant species broad scientific substantiation exists. For example, ESCOP monographs are available for *Linum usitatissimum* L., *Pimpinella anisum* L., *Cetraria islandica* (L.) ACH., *Primula veris* L. and *Salix* ssp..

Complexity of varying chemistry

It is important to consider that the amount of active constituents in plants depends on environmental factors. Based on the plant cultivars used climatic and geographic conditions, quality of the soil and method of cultivation and harvest influence the phytochemical composition of the plant and therefore, different amounts of constituents can be found in different batches. The used part of plant, widely divergent post-harvesting processing and methods of extraction and stabilization affect the chemistry of phytopharmaceuticals [56]. Environmental factors and post harvesting procedures are likely to explain varying effectiveness of a medicinal plant in different studies as reported for Echinacea [57, 58]. Therefore, a direct comparison of the outcome of the studies is difficult especially because of the lack of information regarding the phytochemical composition of the used test material. The use of pharmacopoeia quality in future research would ensure a defined amount of active constituents [59].

Table 6 Promising medicinal plants for treatment and prophylaxis of gastrointestinal and respiratory diseases and for modulation of the immune system and inflammation

Indication	Traditional applications in *initial sources*[1] (number of references recommending the plant for the indication)[a]	Peer-reviewed references (sum of points gathered in the scoring system[2])[b]
Gastrointestinal tract	***Matricaria recutita* L.** (7)	*Allium sativum* L. (41)
	***Foeniculum vulgare* (L.) MILL.** (6)	***Mentha x piperita* L.** (30)
	Potentilla erecta (L.) RAEUSCH (5)	***Salvia officinalis* L.** (27)
	Linum usitatissimum L. (5)	*Origanum vulgare* L. (24)
	Rubus fruticosus L. (5)	***Camellia sinensis* (L.) KUNTZE** (18)
	***Thymus vulgaris* L.** (4)	***Matricaria recutita* L.** (15)
	Quercus robur L. (4)	*Glycyrrhiza glabra* L. (14)
	***Mentha x piperita* L.** (4)	***Thymus vulgaris* L.** (13)
	***Urtica dioica* L.** (4)	***Foeniculum vulgare* (L.) MILL.** (7)
	Vaccinium myrtillus L. (4)	***Carum carvi* L.** (6)
	***Salvia officinalis* L.** (4)	*Pimpinella anisum* L. (6)
	***Carum carvi* L.** (4)	*Rumex sp.* L. (6)
	***Camellia sinensis* (L.) KUNTZE** (4)	***Urtica dioica* L.** (6)
	Achillea millefolium L. (4)	
Respiratory tract	***Thymus vulgaris* L.** (7)	*Echinacea purpurea* (L.) MOENCH (10)
	Pimpinella anisum L. (6)	***Thymus vulgaris* L.** (10)
	***Althaea officinalis* L.** (5)	***Althaea officinalis* L.** (8)
	Cetraria islandica (L.) ACH. (5)	*Glycyrrhiza glabra* L. (5)
	Primula veris L. (5)	*Salvia officinalis* L. (5)
	Foeniculum vulgare (L.) MILL. (4)	*Tussilago farfara L.* (5)
	***Sambucus nigra* L.** (4)	*Urtica dioica* L. (5)
	Malva sylvestris L. (4)	*Achillea millefolium* L. (4)
	Allium sativum L. (4)	*Camellia sinensis* (L.) KUNTZE (4)
	Picea abies (L.) H.KARST. (4)	***Sambucus nigra* L.** (4)
Modulation of immune system and inflammation	***Echinacea purpurea* (L.) MOENCH** (2)	***Echinacea purpurea* (L.) MOENCH** (41)
	Salix sp. L. (2)	*Camellia sinensis* (L.) KUNTZE (26)
	Thymus vulgaris L. (1)	*Glycyrrhiza glabra* L. (19)
	Sambucus nigra L. (1)	*Origanum vulgare* L. (19)
	Urtica dioica L. (1)	***Allium sativum* L.** (18)
	***Malva sylvestris* L.** (1)	*Salvia officinalis* L. (16)
	Plantago lanceolata L. (1)	*Urtica dioica* L. (15)
	***Allium sativum* L.** (1)	*Foeniculum vulgare* (L.) MILL. (11)
	Tilia cordata MILL/*Tilia platyphyllos* SCOP. (1)	*Matricaria recutita* L. (11)
	Artemisia absynthum L. (1)	***Malva sylvestris* L.** (9)
	Verbascum sp. L. (1)	*Rumex* sp. L. (9)
	Armoracia rusticana PH. GÄRTN.*(1)*	

[1]initial sources = standard literature, based on traditional empiric knowledge and historical literature of veterinary [42–45], and human phytotherapy [46], peer-reviewed publications of European [47] and Swiss ethnoveterinary medicine [36, 38] and a report of the European Food Safety Authority (EFSA) [48] focusing on the use of plants as feed additives in animal production; [2]Score = 3 x (number of proven effects in clinical studies – number disproof of effects in clinical studies) + 2 x (number of proven effects in *in vivo* studies - number of disproof of effects in *in vivo* studies) + 1 x (number of proven effects in *in vitro* studies - number of disproof of effects of *in vitro* studies) [a]gastrointestinal tract: all plant species recommended for QA 4 times or more in the initial *sources*, ordered by incidence of recommendation by different authors as listed in detail in Additional file 2; [a]respiratory tract: all plant species recommended for QR 4 times or more in the *initial sources*, ordered by incidence of recommendation by different authors as listed in detail in Additional file 2; [a]Modulation of immune system and inflammation: includes all plant species recommended for QL 1 time or more in the *initial* sources, ordered by incidence of recommendation by different authors as presented in file 2 in greater detail; [b]gastrointestinal tract: includes all plant species gathered for QA with a minimum score of 6 ordered by sum of points the plant species gathered; [b]respiratory tract: includes all plant species gathered for QR with a minimum score of 4, ordered by sum of points the plant species gathered; [b]Modulation of immune system and inflammation: includes all plant species gathered for QL with a minimum score of 9, ordered by sum of points the plant species gathered; **bold letters** = plant species recommended in *initial sources* and in peer-reviewed references for same indication

Relevance for the treatment of livestock diseases

From the 418 references assessed, 46 focused on livestock including 19 clinical references for pigs and five for cattle. Most of these clinical studies used the plants as a feed additive and not as a pharmaceutical. This might be due to complex regulatory affairs exacerbating the licensing and authorization of a medicinal plant or a plant extract as a veterinary drug.

Due to missing information regarding the absorption of orally administered medicinal plant compounds by the gastrointestinal tract, local treatment of gastrointestinal diseases might promising compared to a systemic treatment of respiratory diseases. Effective concentrations of, e.g. essential oils via inhalation may be obtained in the respiratory tract, but it is less practicable when larger herds need to be treated. While pigs as monogastrics might be compared with humans, calves are young ruminants and the biotransformation of plants secondary metabolites in the forestomach is not well known. However, in suckling calves plant extracts can be administered by daily milk diet to ensure bypassing the forestomach by oesophagal groove reflex.

We identified only a few recent references and also few traditional recommendations for the indication QR ($n = 57$) compared to QA ($n = 198$). Interestingly, similar findings were reported from ethnoveterinary research [47, 60]. One explanation might include the challenge of the treatment of respiratory diseases because systemic effects are needed to obtain the therapeutic effect compared to primarily local effects. Modulation of the immature or deficient immune system of calves and piglets provides a starting-point for the prevention of multifactorial infectious diseases. In the traditional phytotherapy literature, effects of medicinal plants on the immune system cannot be found frequently. This might be explained by the fact that immunology is a relatively young scientific field that developed rapidly in recent years. In human medicine, some immune stimulating preparations are already available on the market and therefore, a variety of studies is available. In contrast, for livestock, scientific knowledge is not transferred to practical use yet.

This review mainly focused on therapeutic options of medicinal plants. From this point of view, the relevance of possible toxicity, adverse effects or residues in livestock products remains open. Regarding safety aspects *Tussilago farfara* L. cannot be recommended as a therapeutic medicinal plant due to the presence of toxic pyrrolizidine alkaloids. Nevertheless, the majority of plant species in this review are consumed by humans as food, spices, luxury foodstuffs or as registered nutraceuticals and pharmaceuticals. If these plant species are safe for ingestion in humans, it might be legitimate to transfer these results to other mammalians with a comparable metabolism (herbivores and omnivores). Under these circumstances, risks for humans based on residues in products from food-producing animals should be neglectable. For herbivores and omnivores with a mainly plant-based ration, safety of the most medicinal plant species can be supposed. These species may cope with plant secondary metabolites in a similar way as humans [35].

Promising plant species for gastrointestinal and respiratory diseases and for modulation of the immune system and inflammation

Several trials show the equivalence of plant-derived pharmaceuticals with synthetic ones, but nonetheless there are some trials showing the contrary. Based on the data presented in this review, *Allium sativum* L., *Mentha x piperita* L. and *Salvia officinalis* L. carry a high potential for treatment of gastrointestinal diseases (Table 3). *Echinacea purpurea* (L.) MOENCH, *Thymus vulgaris* L. and *Althaea officinalis* L. may be considered for the treatment of respiratory diseases (Table 4). Regarding the majority of positive results of studies evaluated, *Echinacea purpurea* (L.) MOENCH, *Camellia sinensis* (L.) KUNTZE and *Glycyrrhiza glabra* L. were found to stimulate the immune response (Table 5).

Traditionally, plant species with a high content of tanning agents are administered in diarrhea. *Allium sativum* L. does not contain tanning agents, but due to its antibacterial, antidiarrheal, anti-inflammatory and immunomodulatory effects, it may be used for prophylactic and acute treatment in diarrhea of calves and piglets. Eight *in vivo* and *clinical* studies were identified for *Allium sativum* L. proving these effects, and no studies disproving them. A trial conducted with neonatal calves showed that allicin, a main active compound of *Allium sativum* L., delayed the onset of diarrhea due to *Cryptosporidium parvum* [61]. Two clinical studies demonstrated antidiarrheal effects and a reduction of the fecal coliform count by *Allium sativum L.*. There is also evidence for an improvement of performance in pre-ruminant calves [62, 63]. The immunomodulatory activity of *Allium sativum* L. in pigs [64, 65] and poultry [66, 67] may hold true in immunocompromised calves to support their immune defense. Its antibacterial effects on *Escherichia coli* and *Salmonella* ssp. *in vitro* suggest a high probability of antibacterial activity *in vivo* [68–75]. Nevertheless, more clinical studies are necessary to investigate antiinfective effects of *Allium sativum* L. in young farm animals. *Allium sativum* L. has been reported to exhibit anti-inflammatory activity in rats [76]. There is also mechanistic evidence for anti-inflammatory properties as well as immunostimulation showed in three in *vitro studies*, namely an inhibition of leucocyte migration [77], modulation of interleukin and interferon-gamma expression [78] and a suppression of nitrogen oxide production in macrophages [79]. An anti-inflammatory effect may be useful for the treatment of systemic inflammation

processes often accompanied with diarrhea. Facing animal welfare and regarding efficient synthetic non-steroidal and steroidal anti-inflammatory agents, it is debatable whether there is a need for plant-derived alternatives. While synthetic non-steroidal and steroidal anti-inflammatory agents often produce considerable adverse effects including an inhibition of mucus production [80, 81], medicinal plants compass considerable adverse-effects because they contain several different active compounds which might reduce the potential of unwanted effects [82]. In *in vivo* tests for acute and chronic toxicity, the maximum tolerance dose and genotoxicity *Allium sativum* L. was demonstrated to be relatively safe if administered in therapeutic dosages [83] and if estimated for the animals metabolic body weight [84]. With respect to food quality, it must be assured that residues of *Allium sativum* L., responsible for the typical taste of this plant do not result in an altered taste of meat.

To reduce enteral spasm during diarrheal diseases, *Mentha x piperita* L. might be an efficient treatment option based on three clinical studies in humans, demonstrating efficient spasmolytic activity comparable to butylscopolamine [85–87]. The underlying mechanism includes inhibition of smooth muscle contractility through the block of calcium influx by menthol [88]. In traditional medicine, *Mentha x piperita* L. has been used in the therapy of respiratory diseases. Peppermint essential oil showed spasmolytic activity on rat trachea *ex vivo* [89]. But there are no clinical studies in veterinary medicine for *Mentha x piperita* L. in respiratory disease. No adverse effects have been reported for *Mentha x piperita* L. infusions or oral intake of leaves [90]. Excessive inhalation or local application of pure *Mentha x piperita* L. essential oil was shown to lead to hypersensitivity reactions [56]. Contraindications are severe hepatic damage and cholestasis [43].

Based on this review, the most prominent plant species for stimulation of the immune system is *Echinacea purpurea* (L.) MOENCH. The main constituents are polysaccharides, alkylamides, caffeic acid esters and polyacetylenes [91]. It has been used in therapy for stimulation of the immune system in human medicine, mainly for prevention of viral infections of the respiratory tract [92]. A total of 23 clinical and *in vivo* studies revealed multiple effects on the innate and acquired immune system. *Echinacea purpurea* (L.) MOENCH was shown to increase the immune response towards swine erysipelas vaccination in piglets [93], prevented enveloped virus infections in humans [94] and reduced symptom severity in naturally acquired upper respiratory tract infections in humans [95]. In contrast, seven clinical or *in vivo* studies reported the absence of the above mentioned effects. For example *Echinacea purpurea* (L.) MOENCH failed to enhance growth or to show any immunomodulatory effect in one study in pigs [96]. Reasons for these negative results may be due to a very small number of individuals, an improper dosage or study design. As mentioned above, the diversity of non-standardized *Echinacea* preparations with varying chemistry is likely to result in different findings. In some studies, the dosage was not reported, and therefore it was not possible to estimate how much plant material or drug equivalent was administered per day. Consequently, due to missing data final conclusions cannot be drawn. Nevertheless, eight *in vivo* studies reported modulations of immune system and blood cell count, and no studies were found disproving these effects. Different Echinacea species were found to increase the total number of white and red blood cells in mice [97] and horses [98]. Twenty-six *in vitro* studies demonstrated the underlying mechanisms of immunomodulatory effects of *Echinacea purpurea* (L.) MOENCH. It was reported to activate macrophages and natural killer cells [99–101] and to modulate several cytokines [102–105]. *Echinacea purpurea* (L.) MOENCH is known as a safe immunostimulant in humans and several products are available on the market. No reported interactions with other drugs and no toxic effects after overdosage were reported [106]. Possible rare adverse effects such as hypersensitivity reactions are reported, but no adverse effects have been observed during long-term administration [107]. In general, *Echinacea purpurea* (L.) MOENCH seems to be effective in preventing respiratory diseases and as an early intervention immediately after onset of first symptoms of infectious diseases [95]. However, further veterinary clinical studies need to be performed, especially to evaluate effective dosages.

In human medicine, *Thymus vulgaris* L. has been already effectively used according to its antitussive and mucolytic effects in the treatment of acute bronchitis, often in combinations with other plant species, e. g. *Primula veris* L. for its expectorant effects [108–110]. The main active compound of *Thymus vulgaris* L. is the essential oil containing thymol, geraniol, thujanol and linalool [111]. The above mentioned effects still have to be investigated for veterinary purposes. Nevertheless, an enhancement of the mucociliary clearance in mice was shown in two *in vivo* studies [112, 113]. This effect was explained by an interaction with $beta_2$ receptors in rat lung tissue [112]. Additionally, three *ex vivo* studies demonstrated spasmolytic effects of *Thymus vulgaris* L. on tracheal chains comparable to theophylline [114–116]. The reported anti-inflammatory properties [117] and antibacterial effects found in *in vitro* studies [118–120] of *Thymus vulgaris* L. still needs to be investigated in clinical studies. *In vivo* studies on the toxicology of *Thymus vulgaris* L. leaf extract showed no toxic potential [121, 122]. In summary, the data available support the potential for using *Thymus vulgaris* L. for treatment of respiratory diseases in livestock.

Camellia sinensis (L.) KUNTZE seems to be useful for treatment of diarrhea as well as for stimulation of the immune system. Main constituents are polyphenolic compounds (up to 25 % catechin derivatives in non-fermented

plants, e.g. epigallocatechin), purine alkaloids (caffeine, theobromine, theophylline) and flavonoids [123]. Some clinical studies reported beneficial effects of *Camellia sinensis* (L.) KUNTZE on gut health as indicated by a reduced prevalence of postweaning diarrhea in piglets, but also a decrease in growth performance [124]. An experimental trial on a diet with *Camellia sinensis* (L.) KUNTZE whole plant extract revealed a significant decrease of *Clostridia* counts, but also of *Enterococci* counts in the feces of piglets compared to a standard diet with antibiotics [125]. Two *in vivo* studies showed also anti-influenza virus activity in mice [126] and chicken [127], which might be due to an inhibition of virus adsorption [126]. Fifteen *in vitro* and five *in vivo* studies demonstrated antioxidative [128–130] and anti-inflammatory [131, 132] effects and a modulation of the immune system [133, 134]. No studies were found disproving these effects. In mice, the intake of a concentrated extract of *Camellia sinensis* (L.) KUNTZE did not lead to unwanted adverse effects [135]. Despite that *Camellia sinensis* (L.) KUNTZE is known to be fairly devoid of unwanted effects, some reports on liver damage related to the intake of *Camellia sinensis* (L.) KUNTZE extract are available [136, 137]. In summary, most reports state that safety of *Camellia sinensis* (L.) KUNTZE extract can be supposed, if used appropriately to the recommendations [136]. Therefore, *Camellia sinensis* (L.) KUNTZE bears a reliable potential for prophylaxis and therapy of diseases in calves and piglets.

The main active compound of medicinally used roots of *Glycyrrhiza glabra* L. is the saponin glycyrrhizin. Furthermore, it contains flavonoids and isoflavonoids, chalcones, cumarins and phytosterols [123]. With regard to the inclusion criteria of this review, no clinical studies focusing on *Glycyrrhiza glabra* L. could be found. Nevertheless, it was shown to exhibit immunostimulatory effects *in vivo*, by stimulation of cellular and nonspecific response [138]. In three *in vivo* and *ex vivo* models, antitussive [139] and tracheal smooth muscle relaxing activity [140] as well as regulating effects in the gastrointestinal tract were reported [141]. Four *in vitro* studies demonstrated its antimicrobial [142, 143] and antiviral potential by activation of autophagy [144]. In an assessment of different plant species, *Glycyrrhiza glabra* L. exerted the strongest antiviral activity against rotavirus [145]. Due to these versatile effects, *Glycyrrhiza glabra* L. might be beneficial for prophylaxis and treatment of virus induced diseases of the respiratory- as well as the gastrointestinal tract. Regarding the safety of *Glycyrrhiza glabra* L., it is important to choose the right dosage due to the hyper-mineralocorticoid-like effects of glycyrrhizin. An acceptable daily dosage of 0.015–0.229 mg glycyrrhizin/kg body weight/day for human and animals was reported [146].

Conclusions

This systematic review identified common medicinal plant species as a potential future therapeutic option for gastrointestinal and respiratory diseases in calves and piglets. Based on their plant specific multi-component compositions, the versatile effects of medicinal plants as 'multi-target drugs' may bear a potential for the treatment of respiratory and gastrointestinal diseases in calves and piglets. Medicinal plants are unlikely to replace chemical medications as a general rule, but they may be a single or at least a complementary treatment. In concert with housing, feeding and hygiene, medicinal plants are part of a sustainable, natural option for improving animal health and reducing the use of antimicrobials in livestock farming. The results of this review provide support for a need for additional *in vitro, in vivo* and clinical research focused on phytotherapy for recently emerging and challenging diseases in livestock. While a large amount of peer-reviewed studies about medicinal plant species is available, most of the clinical and experimental studies were performed in humans and experimental animals. More research is needed to evaluate the potential of medicinal plants for treatment of farm animals. The data from this review provide guidance on medicinal plants promising for further investigations in livestock: the most promising candidates for gastrointestinal diseases are *Allium sativum* L., *Mentha x piperita* L. and *Salvia officinalis* L.; for diseases of the respiratory tract, *Echinacea purpurea* (L.) MOENCH, *Thymus vulgaris* L. and *Althea officinalis* L. were found most promising, and *Echinacea purpurea* (L.) MOENCH, *Camellia sinensis* (L.) KUNTZE, *Glycyrrhiza glabra* L. and *Origanum vulgare* L. were identified as best candidates for modulation of the immune system and inflammation. Based on this review, studies are under way to investigate the effects of promising medicinal plants in calves and piglets.

Additional files

Additional file 1: Protocol of the systematic review.

Additional file 2: Plant species recommended for the treatment of gastrointestinal and respiratory diseases in human and animals based on standard textbooks, European peer-reviewed ethnoveterinary publications and an EFSA report.

Additional file 3: Assessment of medicinal plants effects in 418 final peer-reviewed references.

Abbreviations

ATCvet, Anatomical Therapeutic Chemical classification system for veterinary medicine; e.g., *exempli gratia* (in English: for example); EFSA, European Food Safety Authority; EMA, European Medicines Agency; ESCOP, European Scientific Cooperative On Phytotherapy; MBW, metabolic body weight; pH, pondus Hydrogenii; PICOS, population, intervention, comparator, outcome, study design; QA, preparations used for the treatment of diseases affecting the alimentary tract or metabolism, particularly unspecified or infectious diarrhea and intestinal spasms; QL, immunomodulating agents; QR, preparations for the treatment of diseases in the respiratory system; sp., any species, not specified in detail; ssp., subspecies; WHO, World Health Organization

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Authors' contributions
HA designed the review, collected, analysed and interpreted the data and wrote the manuscript. MMev participated in the design of the study, in the interpretation and discussion of data and coordinated the writing and reviewing of the manuscript. MK contributed his expert-knowledge on diseases of calves and participated in the interpretation and discussion of data. HN and NG contributed their expert-knowledge on diseases of piglets and participated in the interpretation and discussion of data. MMel contributed his expert-knowledge on pharmaceutical biology and participated in the interpretation and discussion of data. MW participated in the design of the study, in the interpretation and discussion of data and in the writing of the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests'.

Consent for publication
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Molecular iodine/doxorubicin neoadjuvant treatment impair invasive capacity and attenuate side effect in canine mammary cancer

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Abstract

Background: Mammary cancer has a high incidence in canines and is an excellent model of spontaneous carcinogenesis. Molecular iodine (I_2) exerts antineoplastic effects on different cancer cells activating re-differentiation pathways. In co-administration with anthracyclines, I_2 impairs chemoresistance installation and prevents the severity of side effects generated by these antineoplastic drugs. This study is a random and double-blind protocol that analyzes the impact of I_2 (10 mg/day) in two administration schemes of Doxorubicin (DOX; 30 mg/m2) in 27 canine patients with cancer of the mammary gland. The standard scheme (sDOX) includes four cycles of DOX administered intravenously for 20 min every 21 days, while the modified scheme (mDOX) consists of more frequent chemotherapy (four cycles every 15 days) with slow infusion (60 min). In both schemes, I_2 or placebo (colored water) was supplemented daily throughout the treatment.

Results: mDOX attenuated the severity of adverse events (VCOG-CTCAE) in comparison with the sDOX group. The overall tumor response rate (RECIST criteria) for all dogs was 18% (interval of reduction 48–125%), and no significant difference was found between groups. I_2 supplementation enhances the antineoplastic effect in mDOX, exhibiting a significant decrease in the tumor epithelial fraction, diminished expression of chemoresistance (MDR1 and Survivin) and invasion (uPA) markers and enhanced expression of the differentiation factor known as peroxisome proliferator-activated receptors type gamma (PPARγ). Significant tumor lymphocytic infiltration was also observed in both I_2-supplemented groups. The ten-month survival analysis showed that the entire I_2 supplementation (before and after surgery) induced 67–73% of disease-free survival, whereas supplementation in the last period (only after surgery) produced 50% in both schemes.

Conclusions: The mDOX+I_2 scheme improves the therapeutic outcome, diminishes the invasive capacity, attenuates the adverse events and increases disease-free survival. These data led us to propose mDOX+I_2 as an effective treatment for canine mammary cancer.

Keywords: Canine mammary cancer, Molecular iodine, Neoadjuvant chemotherapy, Doxorubicin, Animal welfare

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Background

Mammary cancer is the neoplasia with most incidences in intact female dogs [1]. Currently, surgery remains the mainstay of canine mammary cancer treatment, but several studies describe adjuvant chemotherapy as an approach that increases quality of life and disease-free survival [2–5]. In women, the use of systemic chemotherapy before local surgical excision, known as neoadjuvant therapy, may also reduce the extent of local surgery without jeopardizing patient survival and disease-free survival [6]. This practice is still scarce in dogs, but recent reports describe the use of this approach to transform unresectable tumors into resectable ones even though disease-free survival or survival time was not significantly longer when different chemotherapeutic drugs were used as monotherapy [7–9].

One of the most important considerations in chemotherapy treatment is the attenuation of toxicity and chemoresistant effects. Slow infusion is one of the most explored strategies to reduce toxicity. Doxorubicin (DOX), a widely used therapeutic drug, exhibited a direct correlation between its toxic effects and its peak plasma concentration. Rapid intravenous bolus results in higher peak levels, whereas slow infusion rates lead to a greater area under the curve associated with less toxicity. However, the pharmacokinetics of this drug could be different between species. In humans, slow infusion (1 to 6 h or more) exhibited less toxicity in cardiac tissue and other organs [10], whereas in rodents fast infusion (5 versus 30 min) seems to be better protection [11]. Related studies in canines were not available until now. A second strategy, more represented in canine studies, is the use of chemotherapeutic drugs in different schemes or pharmaceutical presentations (metronomic therapy, liposomes, nanoparticles) [12–15], specific molecules that act as inhibitors of growth factor receptors (toceranib phosphate) [16, 17], components that exacerbate immune anti-tumor responses (anti Her2, TNFα) [18, 19] or even natural products with adjuvant antineoplastic effects (Curcumin) [20].

In this regard, previous work by several research groups has shown that molecular iodine (I_2) exerts antineoplastic effects on different cancer cells grown under basal, chemoresistant and stem (spheroid) conditions by activating re-differentiation pathways and reversing chemoresistance and epithelial-mesenchymal transition (EMT) [21–23]. Similarly, pre-clinic (immunosuppressed mice and pharmacologically induced rats) and clinic mammary cancer protocols have shown that I_2 supplementation inhibits tumor growth, activates the antitumor immune system and exerts antioxidant actions that prevent the severity of side effects generated by antineoplastic drug toxicity [24, 25]. The antineoplastic actions of I_2 include direct mechanisms exerting oxidant effects on the mitochondrial membrane and indirectly generating an iodinated lipid known as 6-iodolactone (6-IL), a specific ligand of peroxisome proliferator-activated receptor gamma (PPARγ) [26]. PPARs are ligand-activated transcription factors, and three subtypes have been identified (PPAR alpha, beta, and gamma). PPARγ is involved in lipid metabolism and has recently been shown to play a significant role in cell proliferation, differentiation, and apoptosis in many types of cancer [27]. The present study was designed to analyze the effect of I_2 supplementation in two DOX administration schedules: standard (21-day cycles; 20 min infusion) versus modified (15-day cycles; 60 min infusion) regimens in canine patients with mammary cancer.

Methods

Patients

The study was performed to include at least between 4 and 10 dogs per group according to our previous studies in rodents [25]. We invited and registered 93 patients who gradually abandoned the protocol for various reasons. Some of them related to the side effects (although we were very attentive to their health and well-being, many owners did not accept the loss of hair or the temporary change of mood), or due to other difficulties (abandonment of treatment, death independent of cancer, pyometra, etc.). As the study was double-blind, the confirmation of the groups was revealed until the day of the surgery and it was not possible to re-adjust them. Twenty-seven intact female dogs with acceptable health and a diagnosis of mammary cancer finished the protocol. All procedures were performed at the Veterinary Hospital of the Universidad Autónoma de Querétaro and at the Veterinary Hospital of FES-Cuautitlán, UNAM. Additionally, all procedures followed the Animal Care and Use program (NIH, USA) and were approved by the Research Ethics Committee of INB-UNAM (Protocol #102).

Clinical protocol

Cytological diagnostic of mammary cancer was performed by a sample of puncture fine needle aspiration. Eligibility criteria also included signed informed consent and normal hepatic, renal and cardiovascular function (general blood and urine analysis and electrocardiogram, respectively). A biopsy was performed in accepted dogs to confirm the cytological diagnosis and determine the tumor classification according to Goldschmidt et al. [28]. A dog health questionnaire was filled out by the veterinary surgeon and the dog owner before every chemotherapy cycle. Toxicity was graded using the Veterinary Cooperative Oncology Group - Common Terminology Criteria for Adverse Events (VCOG-CTCAE) [29]. The clinical signs considered in the present work

were weight, vomitus, diarrhea, anorexia, lethargy, anemia and neutropenia. Distant metastasis disease was identified using ultrasound and thorax radiographs.

Chemotherapy

The standard scheme (sDOX) comprises four cycles of DOX (30 mg/m^2) administered intravenously for 20 min every 21 days, while the modified regimen (mDOX) consists in the same amount of chemotherapy infused for 60 min in four, 15-day cycles.

Molecular iodine

Was prepared with Lugol solution (Golden Bell, Edo. de Mexico, Mx) and diluted in distilled water. Iodine (10 mg/day) or placebo (water with artificial food coloring) was administered orally with daily food 1 week before chemotherapy and continuously until mastectomy. The file for each dog was opened on the day of the mastectomy to identify the subgroup (I_2 or placebo). To analyze the effect of I_2 supplementation on survival and disease-free survival rates, all patients received the halogen supplementation for ten more months.

Mastectomy

The veterinary surgeon carried out the pre-surgical protocol and the corresponding postoperative monitoring during the peri-mastectomy period. The surgery was performed 6 weeks after the last chemotherapy to ensure the complete re-establishment of the patients' health. Anesthesia included premedication with 0.01 mg/kg Acepromazine (Vetoquinol, Mexico City, MX), induction and maintenance with 2 mg/kg Propofol (App pharmaceutics, Los Angeles, CA) and fluid therapy (saline solution). For pain management, 0.005–0.02 μg/kg with Buprenorphine (Schering-Plough, NJ, USA), 0.2 mg/kg Meloxicam (Aranda Lab, Queretaro, MX) and 1 mg/kg Tramadol (Pisa, Mexico City, MX), as well as antibiotic therapy with 5 mg/kg Enrofloxacin (Bayer, Edo de Mexico, MX).

Iodine consumption, thyroid status, cardiac damage and tumor response

All these parameters were recorded on the day that the dogs were accepted into the protocol (initial) and on the day of the mastectomy (final). The content of total iodine in urine was determined by ionic analysis (potentiometer Thermo Scientific Orion Star A214 with ion selective electrode; ISE; LIS-146ICM), triiodothyronine (T3) and thyrotropin (TSH) circulating levels by ELISA (International immuno-diagnostics/IIDE-2021 and Abnova/KA2296, respectively). Serum creatine kinase type MB (CK-MB) activity, used as a marker of cardiac damage, was measured by using the CK-MB Test (Stanbio Laboratory/0980–103). Electrocardiogram (ECG) recordings were made over a minimum 30-s period in each dog, while conscious and lightly restrained, on initial (time 0), 10 min before each chemotherapy session and 1 week before mastectomy. The ECG traces from each animal were examined by a certified veterinary cardiologist to determine the following variables: heart rate, P-wave duration, P-wave amplitude, PR interval, QRS duration, QRS amplitude, QT interval, ST segment structure, and T-wave amplitude.

The tumor response rate was determined according to the Response Evaluation Criteria in Solid Tumors (RECIST) which use only the longest diameters of tumors in the axial plane [30]. The response is placed into one of four categories: complete response (CR; disappearance of all target lesions), partial response (PR; ≥ 50% decrease in sum of target lesions from baseline), stable disease (SD; meets criteria for PR or PD) or progressive disease (PD; ≥ 25% increase in sum of target lesions or appearance of new lesions).

Immunohistochemistry

Was performed on 3 or 5 μm formalin-fixed paraffin-embedded specimens that were float-mounted onto silanized glass slides. ERα was analyzed by a sandwich protocol (polyclonal first antibody HC-20, Santa Cruz Biotechnology. Dallas, Texas, USA; and goat anti-rabbit-immunoglobulin, peroxidase labeled, Dako/K4011). Sections were counterstained with hematoxylin. ERα-positive cells were identified by the presence of a brown stain over the nucleus and cytoplasm. Five regions were analyzed, and tumor samples were considered ERα-positive when 20 or more cells per field were positively labeled in the nucleus, in the cytoplasm or in both, and were found in at least three fields at 40X. Negative controls were obtained by using only the secondary antibody (Dako/K4011). The positive control was performed employing ovary tissue.

Histopathological analysis

Classification and grading of mammary tumors was determined by Goldschmidt et al. criteria which include tubule formation, nuclear pleomorphism and mitoses/hyperchromatism [28]. The histologic malignancy was described as well differentiated (low values), moderately differentiated (intermediate values) and poorly differentiated (high values). Hematoxylin and eosin (H&E) staining was used to observe histological malignancy and lymphocytic infiltration. Masson's trichrome staining was used to identify epithelial (red) and connective (blue) proportions of representative slides per group. Two independent observers evaluated all these characteristics in anonymized and blinded samples. The analysis was performed as the mean of three random regions. The number of lymphocytes and the

percentages of stained areas were calculated using the ImageJ 1.47 program (Wayne Rasband. NIH, USA).

Molecular expression

Bax, Bcl2, urokinase plasminogen activator (uPA), Survivin, multidrug resistance protein 1 (MDR1) and PPARγ expression were analyzed by quantitative real-time PCR (qPCR) [21]. Total RNA was obtained using the TRIZOL reagent (Life Technologies, Inc., Carlsbad, CA), and the extracted RNA (2 μg) was reverse transcribed using oligo-deoxythymidine. qPCR was performed with SYBR green dye (Thermo Scientific/K0221) in detector system Roto-Gene 3000 (Corbett Research, Mortlake, NSW, Australia) with the canine gene-specific primers listed in Table 1. Validation of qPCR was performed using β-actin and phosphoglycerate kinase 1 (PGK1) as non-regulated housekeeping genes. Gene expression was calculated using the D cycle threshold method and normalized to β-actin content.

Statistical analysis

All statistical analysis was performed in Prism version 6.01 (GraphPad Software). An unpaired, Student's *t*-test was used to compare average (initial and final) of total urine iodine, serum thyroid hormones and cardiac enzyme (CK-MB). Unpaired one-way ANOVAs were used to analyze electrocardiogram profile, residual tumor size and epithelial/connective tissue proportion. The differential gene response was performed with Mann-Whitney U, and Pearson correlation was used to determine correlations between residual tumor size and lymphocyte number. A p value of less than 0.05 was considered significant.

Results

Table 2 summarizes the patients' characteristics. The median age was 9.2 ± 2.4 years, and the most affected breed was the Standard Poodle (33%). Sixty-eight percent of dogs were nulliparous, and none received hormone therapy. Only 20% (6 dogs) of the patients exhibited overweight (between 10% and 30% above standard weight). Two patients presented distant metastasis (lungs) at the time of diagnosis (clinical state V).

Grading of adverse events for each group throughout the treatment is summarized in Fig. 1. Patients started with excellent conditions (0), and lower and medium grades (1 to 2) were observed after the second or third chemotherapy cycle (before the third or fourth chemotherapy cycle, respectively). No patients exhibited significant weight loss or developed grades 3, 4 or 5 (death) during treatments. Table 3 describes the number of patients who presented clinical signs and their severity (grade) at any time during chemotherapy treatment. The mDOX scheme reduces the severity of the general symptoms in comparison with sDOX. The I_2 supplement further attenuates the severity of the symptoms in both treatments. The six dogs that presented grade 2 vomiting and/or diarrhea were complemented with supportive care: antibiotic (Metronidazole, 30–40 mg/kg for 14 days) and probiotics (Enterogermina 2 billion/5 mL); with Omeprazole (20 mg/kg for 24 h), Ranitidine (3 mg/kg every 12 h for 8 days), Difenidol (1 mg/kg for 4 days), and/or fluid therapy (saline solution).

I_2 ingestion, thyroid status and cardiac damage were recorded when patients were accepted into the protocol (initial) and on the day of mastectomy (final). Total urinary iodine values were above 2000 μg/L in supplemented patients and DOX groups did not show any changes. I_2 supplementation and/or DOX administration neither modified T3 or TSH circulating values or showed cardiac damage measured by serum CK-MB activity (Fig. 2). The electrocardiogram variables from initial (time 0) and final (before mastectomy) are summarized in Fig. 3. No significant changes were observed between groups in any variable or protocol day.

Figure 4 shows the tumor classification, histological grade and residual tumor size. Most patients developed more than one tumor (Table 2). The tumors with enough size (more than 1 cm^3) were analyzed as individual tumors. Ninety-one percent were of the epithelial type, whereas only 9% corresponded to the mixed type (carcinosarcoma). Histological grading showed that 60%

Table 1 Oligonucleotides

Gen	Reference	Forward primer	Reverse primer	pb
β-actin [1]	NM_001101	acagagtacttgcgctcagga	ccatcatgaagtgtgacgttg	175
PGK1 [2]	NM_053291.3	tgactttggacaagctggacgtga	cagcagccttgatcctttggttgt	110
Bax [3]	NM_001003011.1	aagctgagcgagtgtctcaagcgc	tcccgccacaaagatggtcacg	366
Bcl2 [4]	NM_000633.2	gtggaggagctcttcaggga	aggcacccagggtgatgcaa	304
PPARγ [5]	XM_005632014.1	ttccattctcaagagcggaccc	tctccacagactcggcattcaa	190
Survivin [6]	NM_001003348.1	accgcgtctctacgttcaag	ccaagtctggctcgttctca	114
uPA [7]	XM_005618862.1	ttggggagatgaagtttgaggtgg	cagaacggatcttcagcaaggc	105
MDR1 [8]	NM_001003215.1	tatcagcagcccacgtcatc	cagccactgctacctacgag	214

[1, 4] Human, [2] Rat, [5, 6, 7, 8] Canine

Table 2 Patient characteristics

Breed	Age (years)	Body weight (kg)	Clinical stage (TNM)φ	Number of tumors	Parturition	ERα
Standard scheme (Dox)						
Standard Poodle	12	6	III	2	0	Positive
Maltese bichon	9	**9.9**	III	7	2	Positive
Dachshund	13	6.6	III	4	2	Positive
Maltese bichon	7	**6.6**	III	5	0	Positive
Standard scheme (Dox + I_2)						
Rottweiler	8	38	III	1	2	Positive
Standard Poodle	12	5.8	II	5	0	Positive
Standard Poodle	10	3.8	III	5	0	Positive
Labrador Retriever	10	30	III	7	0	Positive
Standard Poodle	12	6.4	V	8	1	Positive
Standard Poodle	9	7	III	9	1	Positive
Modified scheme (Dox)						
Cocker Spaniel	9	10.3	II	8	0	Positive
Mixed breed	6	4.2	II	2	0	Positive
Cocker Spaniel	8	13	I	6	3	Positive
Chihuahua	5	2.2	I	3	0	Positive
German Shepherd	7	30.1	III	7	0	Positive
Dalmatian	6	23	I	1	0	Positive
Modified scheme (Dox + I_2)						
Cocker Spaniel	10	**16.8**	III	1	0	Positive
Mixed breed	8	**7.6**	I	5	0	Positive
Standard Poodle	11	4.2	V	7	0	Positive
Cocker Spaniel	13	9.8	III	7	0	Positive
Fox terrier Toy	7	2.4	I	5	0	Positive
Standard Poodle	10	4.5	III	3	0	Positive
Cocker Spaniel	13	**17.4**	III	7	3	Positive
Standard Poodle	10	6	II	8	0	Positive
Standard Poodle	9	4.7	III	6	4	Positive
Chihuahua	5	**3.8**	III	5	0	Positive
Dachshund	10	5.2	III	5	1	Positive

ϕ T – Primary tumor, N – Regional lymph nodes, M – Distant metastasis [28];
Bold numbers, overweight animals (10–30% of standard weight)

of tumors were well differentiated, 37% were moderately differentiated and only one (3%) was poorly differentiated. No dogs exhibited a complete response (CR). One patient from sDOX+I_2 showed partial response (PR) and one from the sDOX group exhibited progressive disease (increases ≥25%). The rest of the tumors (94%) were maintained as stable disease. The overall response rate for all dogs was 18.0%, where the residual tumor size corresponds to 82.0% (interval 48–125%). However, even though tumor response (residual size) did not show statistical differences between groups, I_2 supplementation was accompanied by decreases in epithelial tissue in comparison with connective content (Fig. 5). This observation was significant in the mDOX+I_2 group, suggesting that I_2 acts on all tumor types by increasing the antineoplastic effect of mDOX.

The molecular analyses (Fig. 6) show that I_2 treatment generates similar responses in apoptotic and invasive markers independent of the DOX administration scheme. The presence of I_2 increases the apoptotic index (Bax/Bcl2) and impairs the induction of invasive or chemoresistant genes such as MDR1, uPA and Survivin. The combination of mDOX+I_2 synergies the expression of Bax and PPARγ receptors.

One interesting phenomenon was observed in lymphocytic infiltration. I_2 supplementation increases the

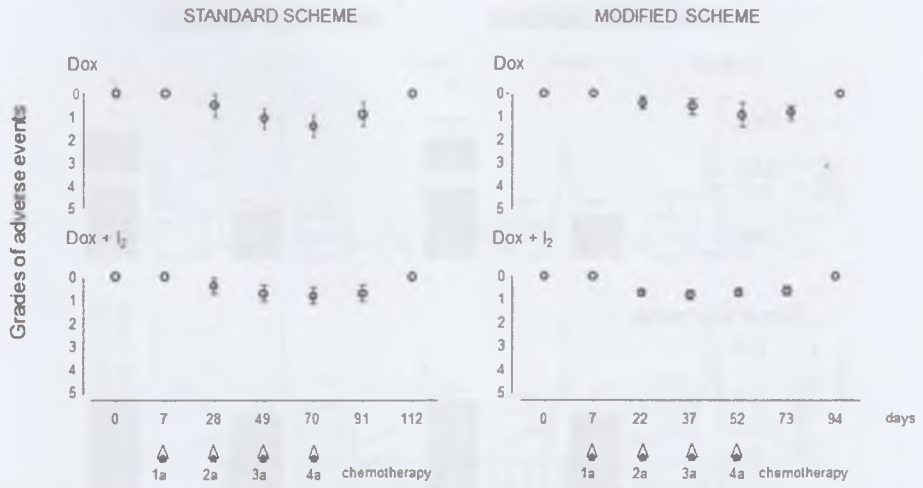

Fig. 1 Functional value according to the VCOG-CTCAE scale. Each point represents the mean and SD for each clinical report during all treatments. Arrows represent the day of chemotherapy (DOX) application

Table 3 Adverse events (VCOG-CTCAE)

	Grade	sDox	sDox + I_2	mDox	mDox + I_2
Vomiting	1	1 (25%)	1 (17%)	1 (17%)	2 (18%)
	2	1 (25%)	1(17%)	1 (17%)	
	3				
	4				
Diarrhea	1	1 (25%)	1 (17%)	1 (17%)	2 (18%)
	2	1 (25%)	1 (17%)	1 (17%)	1 (9%)
	3				
	4				
Anorexia	1	2 (50%)	1 (17%)	1 (17%)	1 (9%)
	2	1 (25%)	1 (17%)		
	3				
	4				
Lethargy	1	1 (25%)	1 (17%)	1 (17%)	1 (9%)
	2	1 (25%)	1 (17%)	1 (17%)	1 (9%)
	3				
	4				
Anemia[a]	1	2 (50%)	2 (33%)	2 (33%)	3 (27%)
	2				
	3				
	4				
Neutropenia	1	1(25%)	1 (17%)	1 (17%)	1 (9%)
	2				
	3				
	4				

[a]Anemia includes: hematocrit, hemoglobin and erythrocyte values (mean globular volume and mean hemoglobin concentration)

presence of lymphocytes in both schemes (Fig. 7) and exhibits a significant inverse correlation with the tumor response (more lymphocytes in the small residual tumor size).

As mentioned before, the use of DOX as monotherapy has the benefit of converting the unresectable tumors into resectable ones, but it does not increase the disease-free survival (~207 days). With this premise, all our patients received I_2 supplementation after the mastectomy, and the survival analysis was performed for ten more months (~300 days). Table 4 shows that I_2 supplementation during the entire treatment (before and after surgery) has an improved disease-free survival of 67 and 73% (sDOX+I_2: 4 dogs and mDOX+I_2: 8 dogs, respectively) compared with the 50% (sDOX: 2 dogs; mDOX: 3 dogs) observed in those that received I_2 only in the last period (after chemotherapy). The two patients with previous metastasis (lungs) received sDOX+I_2 and mDOX +I_2, each. In the first case, metastasis disappeared and after 10 months the patient was still alive without relapse or distant metastasis. In the second case (mDOX +I_2), the metastasis progressed, and the patient died 4 months after surgery. Two patients (in the sDOX+I_2 group) exhibited local mammary cancer relapse 7 months after surgery. Tumors were removed in a second surgery and after 10 months, both were still alive without evident metastasis. Three dogs died from causes independent of mammary cancer (two due to other cancers and one due to pyometra complications).

Discussion

In the present work, we compared two DOX administration schemes and the adjuvant effect of I_2 in canine mammary cancer. Our results showed that both DOX schemes are well tolerated, since no patients exhibited

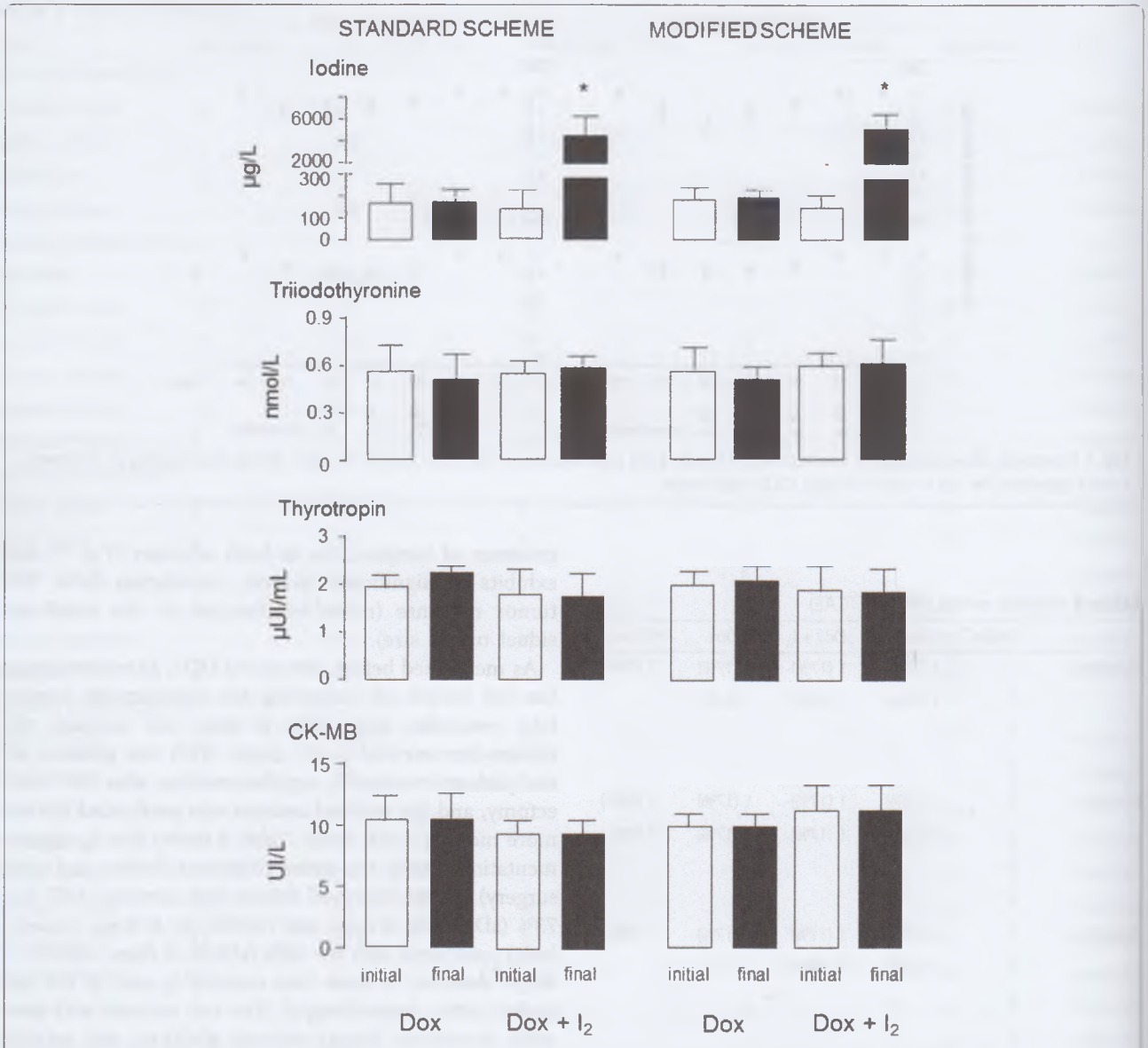

Fig. 2 Iodine ingestion, thyroid status and cardiac damage during treatments. Values were recorded on the day of the patient's admission to the protocol (initial) and on the day of mastectomy (final). Total iodine was determined in urine. Triiodothyronine, thyrotropin and creatine kinase type MB (CK-MB) were quantified in serum. Data are expressed as mean ± SD, and the asterisk indicates a significant difference with respect to the initial condition (unpaired Student t test; $P < 0.05$)

grades 3 or 4, according to the VCOG-CTCAE [29], in both conditions. Moreover, cardiac damage was not evident through serum CK-MB activity or ECG in any group. The clear attenuation of adverse events observed in mDOX agrees with previous reports in humans where slow infusion (1 to 6 h) exhibited less toxicity [10], suggesting that the pharmacokinetics of DOX could be similar between humans and dogs. The addition of the I_2 supplement appears to further attenuate the severity of adverse events. This benefit could be explained by the antioxidant effect of this chemical form of iodine, which is ten times more efficient than ascorbic acid and 100 times more potent than KI (Ferric reducing/antioxidant power assay; FRAP) in vitro [24]. Indeed, previous reports from our group in a rodent mammary cancer model showed that I_2 supplementation prevented weight loss and cardiotoxicity (cardiac lipoperoxidation and serum CK-MB) secondary to DOX, without impairment of its synergistic antitumor action [24].

Regarding the tumor response (RECIST), the overall response was 18% showing that 94% of tumors were maintained as stable disease. This percentage of response is like that described by other authors when chemotherapy drugs are used in the form of

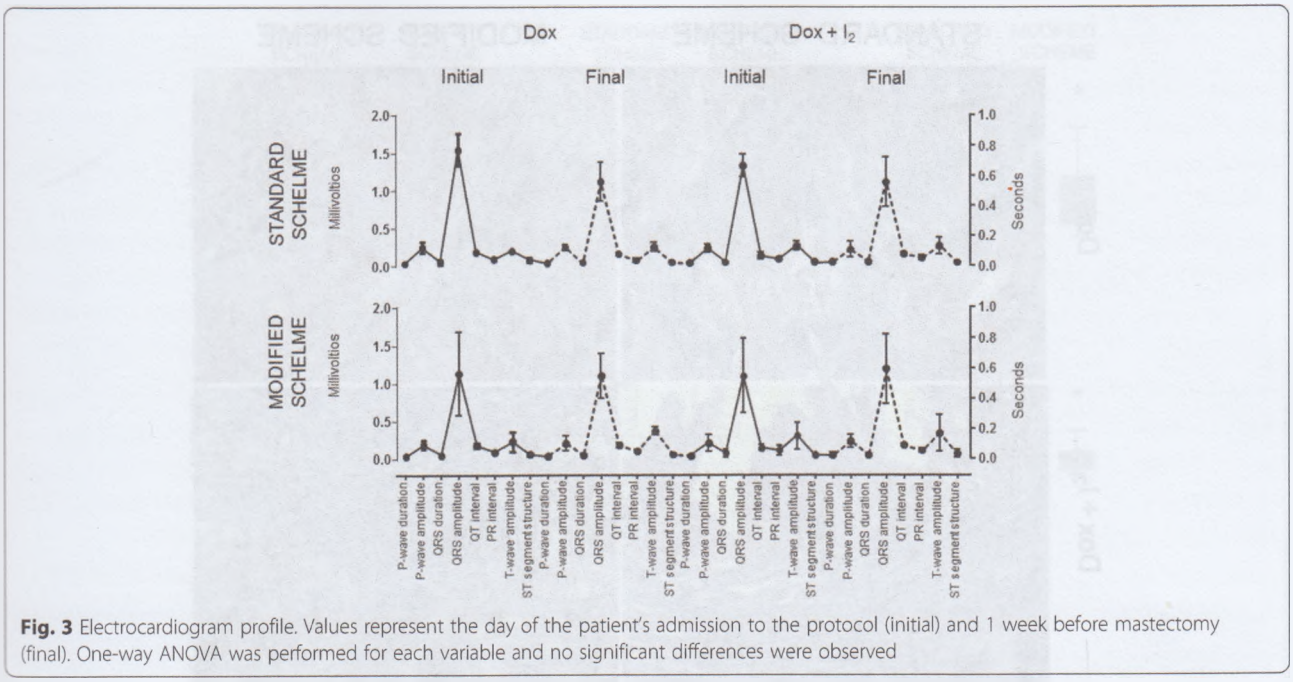

Fig. 3 Electrocardiogram profile. Values represent the day of the patient's admission to the protocol (initial) and 1 week before mastectomy (final). One-way ANOVA was performed for each variable and no significant differences were observed

monotherapy [6–8]; however, the important advantage observed in the I_2-supplemented groups was the significant attenuation of invasive potential in the residual tumor mass. The tumor content showed a significant decrease in epithelial tissue, which is considered the source of tumor progression and metastasis, and a minimum expression of chemoresistance (MDR1 and Survivin) and invasion (uPA) markers, suggesting a better prognosis. It is well established that fibroblast and connective tissue are not I_2-capturing tissues, whereas several types of epithelium uptake this halogen [31]. Moreover, it is described that cancerous cells exhibited between 10 and 100 times more sensitivity to apoptotic I_2 effects than its normal counterpart [22, 31]. Two main pathways have been proposed to explain these effects; one concerning the direct oxidant/antioxidant action of I_2 and a second pathway through iodolipid formation. Upadhyay et al. [32] showed that mitochondria isolated from the tumor (TT) and extra-tumoral tissue (ET) of human breast display significant uptake of iodine; but only TT mitochondria respond by increasing their permeability transition and releasing apoptogenic proteins, indicating a direct and differential proapoptotic action of I_2 in mitochondria from cancerous cells. The second pathway is the generation of an iodinated derivative of arachidonic acid (AA), called 6-iodolactone (6-IL). AA is an essential membrane lipid, and its elevated concentrations in cancerous cells have been implicated in tumoral processes [33, 34]. 6-IL has been detected in tumor mammary glands from rats supplemented with I_2 [35], and this

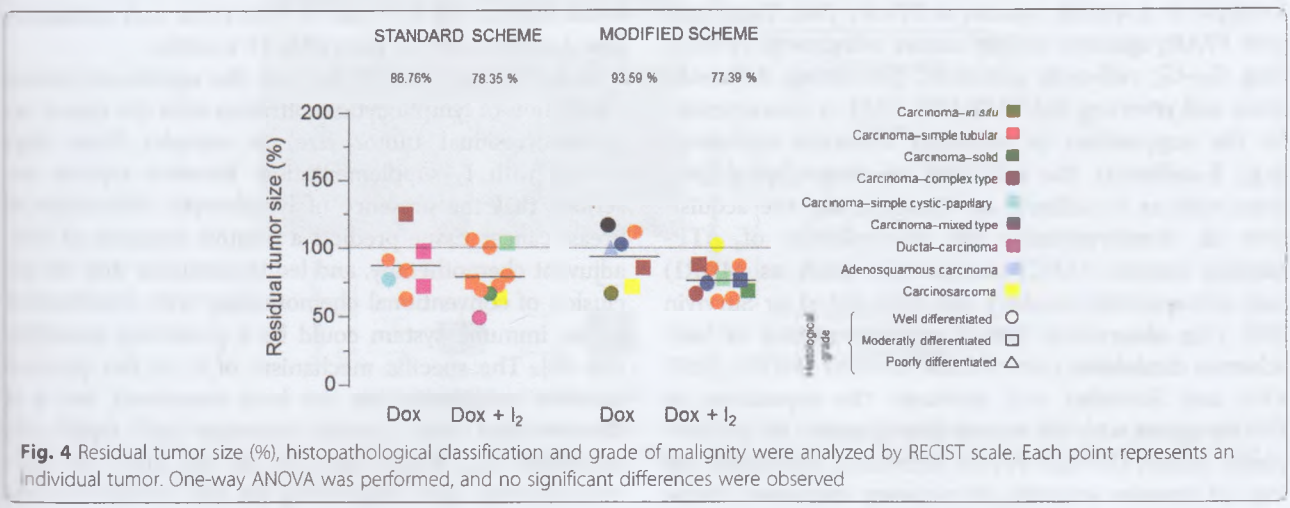

Fig. 4 Residual tumor size (%), histopathological classification and grade of malignity were analyzed by RECIST scale. Each point represents an individual tumor. One-way ANOVA was performed, and no significant differences were observed

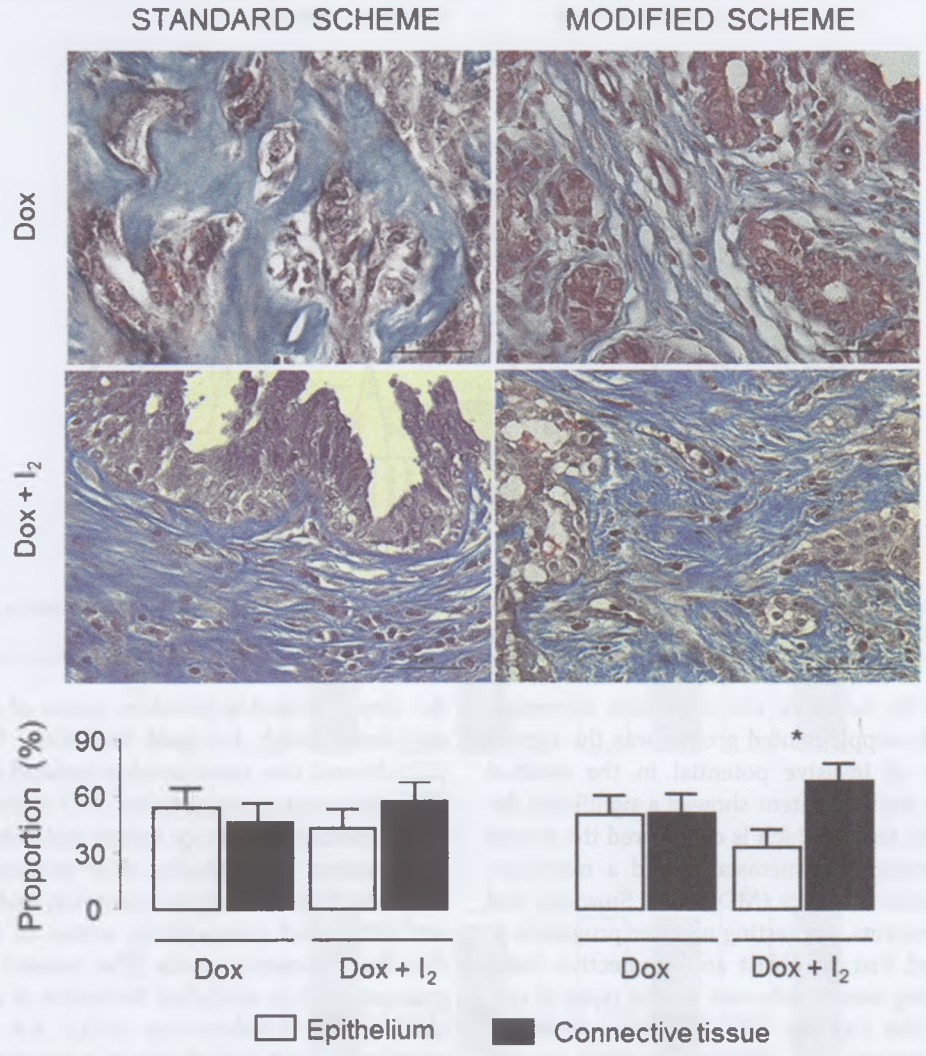

Fig. 5 Epithelial and connective tissue proportion (%) in the final tumor mass. Micrograph staining with Masson's trichrome method (red, epithelium; blue, connective tissue). Quantitative analysis was performed as the average of three random regions (40X) using the ImageJ 1.47 program. Data are expressed as mean ± SD, and the asterisk indicates a significant difference between groups. Student's *t*-test ($P < 0.05$)

iodolipid is a specific agonist of PPARγ [36]. Treatment with PPARγ agonists inhibits cancer cell growth by inducing G_0–G_1 cell-cycle detention, promoting differentiation and reverting the EMT [37]. EMT is characterized by the suppression of adhesion molecule expression (e.g., E-cadherin), the induction of mesenchymal proteins such as N-cadherin or Vimentin and the acquisition of chemoresistance by up-regulation of ATP-binding cassette (ABC) transporters (such as MDR1) and anti-apoptotic markers like Bcl2, Bcl-xl or Survivin [38]. Our observation that I_2 supplementation in both schemes diminishes part of these markers (MDR1, Bcl2, uPA and Survivin) and increases the expression of PPARγ agrees with the notion that I_2 exerts its antineoplastic effects through PPARγ activation. Moreover, the loss of invasive capacity of remnant cancerous tissue could explain the low rate of recurrence and metastasis found in our patients even after 10 months.

Another important finding was the significant inverse correlation of lymphocytic infiltration with the tumor response (residual tumor size) in samples from dogs treated with I_2 supplementation. Previous reports described that the presence of lymphocytic infiltration in breast cancer tissue predicts a positive response of neoadjuvant chemotherapy, and led to speculate that the inclusion of conventional chemotherapy with sensitization of the immune system could be a promising paradigm [39, 40]. The specific mechanism of I_2 in this putative immune modulation has not been elucidated, but it is demonstrated that several immune cell types can internalize I_2, which can act as an anti- or pro-inflammatory agent depending on the cellular context

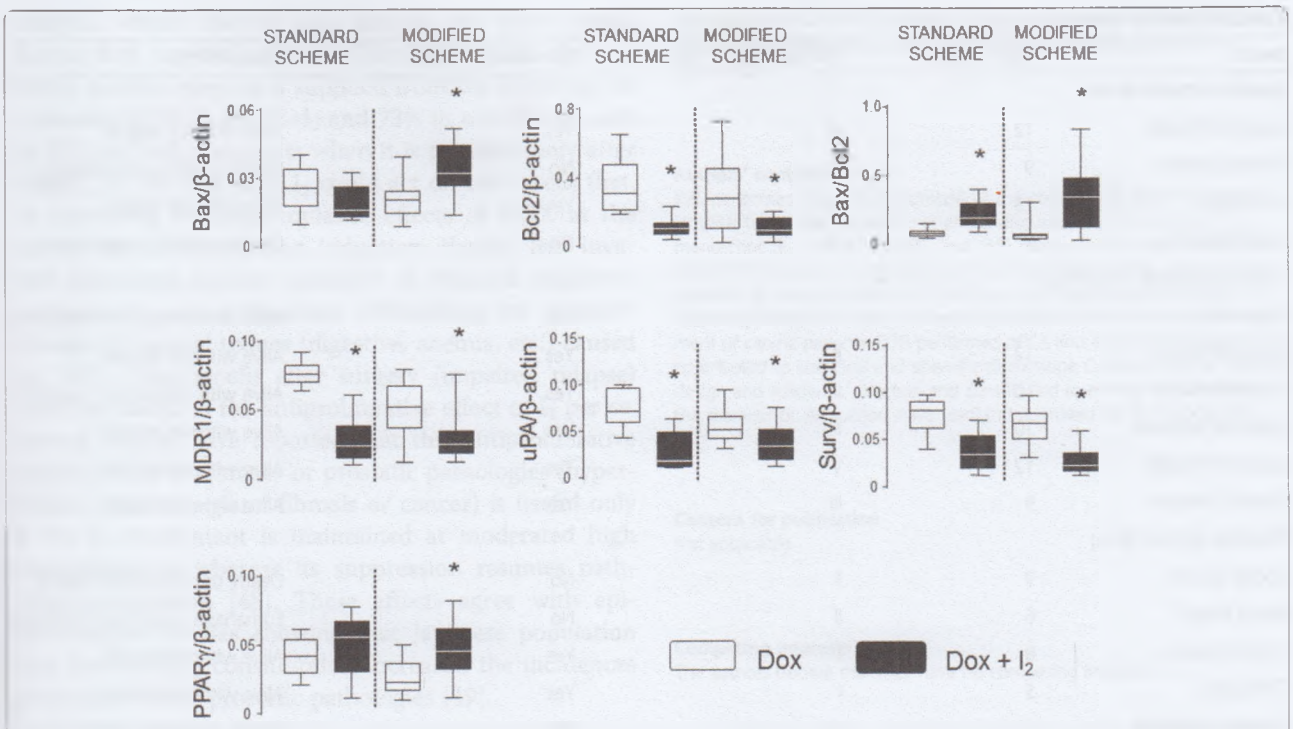

Fig. 6 Effect of treatments on the expression of chemoresistant, invasive and differentiation markers. RT-qPCR amplification were performed in residual tumors. Bax/Bcl2 index as apoptotic induction. Multidrug resistance protein 1 (MDR1); urokinase plasminogen activator (uPA), Survivin protein (Surv). Peroxisome proliferator-activated receptors type gamma (PPARγ). Gene expression was calculated using the D cycle threshold method and normalized to β-actin content. Data are expressed as median and the asterisks indicate significant differences between DOX and DOX + I_2 groups in each treatment (Mann-Whitney U; $P < 0.05$)

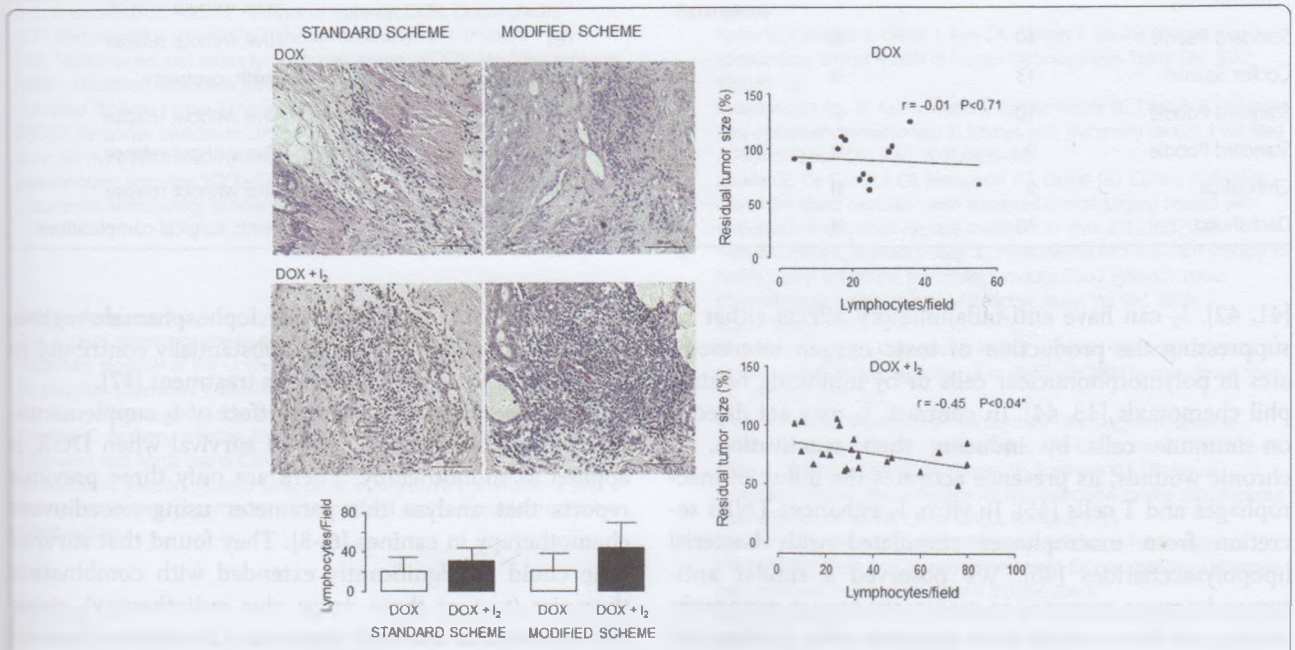

Fig. 7 Effect of treatments on lymphocytic infiltration. Micrographs stained with H&E (20X). Quantitative analysis was performed as the average of three random regions using the ImageJ 1.47 program. Linear regression between residual tumor size (%) and lymphocyte number from DOX and DOX + I_2 groups (* Pearson coefficient, $p > 0.04$)

Table 4 Survival analysis

Breed	Age (years)	Clinical stage (TNM)	Survival at ten months	Observations
Standard scheme (Dox)				
Standard Poodle	12	III	Yes	Alive without relapse
Maltese bichon	9	III	No	Death, surgical complications
Dachshund	13	III	Yes	Alive without relapse
Maltese bichon	7	III	No	Death, surgical complications
Standard scheme (Dox + I_2)				
Rottweiler	8	III	Yes	Alive with relapse; no metastasis
Standard Poodle	12	II	Yes	Alive without relapse
Standard Poodle	10	III	Yes	Alive with relapse; no metastasis
Labrador Retriever	10	III	Yes	Alive without relapse
Standard Poodle	12	V	Yes	Alive without relapse
Standard Poodle	9	III	Yes	Alive without relapse
Modified scheme (Dox)				
Cocker Spaniel	9	II	No	Death, transitional cell cancer
Mixed breed	6	II	No	Euthanasia, suspected metastasis
Cocker Spaniel	8	I	Yes	Alive without relapse
Chihuahua	5	I	Yes	Alive without relapse
German Shepherd	7	III	No	Death, anaplastic cancer
Dalmatian	6	I	Yes	Alive without relapse
Modified scheme (Dox + I_2)				
Cocker Spaniel	10	III	Yes	Alive without relapse
Mixed breed	8	I	Yes	Alive without relapse
Standard Poodle	11	V	No	Death, extensive metastasis
Cocker Spaniel	13	III	Yes	Alive without relapse
Fox terrier Toy	7	I	Yes	Alive without relapse
Standard Poodle	10	III	Yes	Alive without relapse
Cocker Spaniel	13	III	No	Death, pyometra
Standard Poodle	10	II	Yes	Alive without relapse
Standard Poodle	9	III	Yes	Alive without relapse
Chihuahua	5	III	Yes	Alive without relapse
Dachshund	10	III	No	Death, surgical complications

[41, 42]. I_2 can have anti-inflammatory effects either by suppressing the production of toxic oxygen intermediates in polymorphonuclear cells or by inhibiting neutrophil chemotaxis [43, 44]. In contrast, I_2 may act directly on immune cells by inducing their reactivation. In chronic wounds, its presence activates the influx of macrophages and T cells [45]. In vitro, I_2 enhances TNFα secretion from macrophages stimulated with bacterial lipopolysaccharides [46]. We observed a similar antitumor immune response in mammary cancer xenografts when mice Fox1 nu/nu were supplied with I_2 (data not published). Moreover, PPARγ activation could be associated with such effects. In a recent publication, it was demonstrated that PPARγ expression increased almost 5-fold in a metronomic cyclophosphamide regime, suggesting that these receptors substantially contribute to the immune responsiveness of this treatment [47].

Finally, we wanted to test the effect of I_2 supplementation on disease-free-survival and survival when DOX is applied as monotherapy. There are only three previous reports that analyze this parameter using neoadjuvant chemotherapy in canines [6–8]. They found that survival time could be significantly extended with combination therapies (two or three drugs plus radiotherapy), rising the disease-free survival mean to 12 months (interval 213–521 days). In contrast, the disease-free survival only lasts 7 months (217 days) when DOX is used as monotherapy [7]. Our study included a ten-month (~300 days)

analysis, which showed that during this time overall disease-free survival was of 62%. I_2 increases the patient's survival when it is supplied from the beginning of treatment (67% in sDOX+I_2 and 73% in mDOX+I_2), and by 50% in both treatments when it is provided only after surgery. In the first case, I_2 could act on two levels; first, by increasing the antineoplastic effects of DOX at the tumor site (differentiation induction, though less invasive phenotype and/or activation of immune response) and second, as an antioxidant, diminishing the oxidative damage in normal tissues (digestive, anemia, etc.) caused by DOX. The benefit after surgery (impaired relapse) could be linked to the antiproliferative effect of I_2 per se. Several studies have reported that this antiproliferative action of I_2 in mammary or prostatic pathologies (hyperplasia, mammary gland fibrosis or cancer) is useful only if the I_2 supplement is maintained at moderated high concentrations, whereas its suppression resumes pathology progression [48]. These effects agree with epidemiological studies showing that Japanese population that decreased I_2 consumption increased the incidences in mammary and prostatic pathologies [49].

Conclusions

The combination of DOX + I_2 in the modified scheme improved the therapeutic outcome of mammary cancer, increasing both survival rate and relapse-free survival.

Abbreviations

6-IL: 6-iodolactone; ANOVA: Analysis of variance; DOX: Doxorrubicin; ECG: Electrocardiogram; EMT: Epithelial-mesenchymal transition; H&E: Hematoxylin and eosin; I_2: Molecular iodine; mDOX: Modified scheme; MDR1: Multidrug resistance protein 1; PPARγ: Peroxisome proliferator-activated receptors type gamma; qPCR: Quantitative real-time PCR; RECIST: Response evaluation criteria in solid tumors; sDOX: Standard scheme; Surv: Survivin; T3: Triiodothyronine; TSH: Thyrotropin; uPA: Urokinase plasminogen activator; VCOG-CTCAE: Veterinary co-operative oncology group - common terminology criteria for adverse events

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Authors' contributions

XZE performed the clinical protocols as the coordinator of both Veterinary Hospital teams, participated in scientific discussions and wrote the manuscript. BLQ, AADB, MADPC, and GHA performed the staff of Veterinary Hospital FES-Cuautitlan UNAM and were responsible for the care and management of canine patients; BSP, MTM, and LPG performed the faculty of Veterinary Hospital at UAQ and were responsible for the care and management of canine patients. EDG performed qPCR and thyroid status assay. BA contributed to statistical and scientific discussions. CA provided the concept design and academic direction and contributed to editing and drafting of the manuscript. All authors have read and approved the final document.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Pharmacokinetics, bioavailability and dose assessment of Cefquinome against *Escherichia coli* in black swans (*Cygnus atratus*)

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Abstract

Background: The objective of this study is to investigate pharmacokinetics and dose regimens of cefquinome in black swans following intravenous (IV) and intramuscular (IM) administration at a single dose of 2 mg/kg. The MICs of cefquinome against 49 *Escherichia coli* isolates from black swans were determined. Monte Carlo simulation was applied to conduct the dose regimen assessment and optimization of cefquinome against *E. coli* in black swans, and a pharmacokinetic/pharmacodynamic (PK/PD) cutoff was established for *E. coli* isolates obtained in this study.

Results: The PK parameters of $T_{1/2\alpha}$ (0.31 h), $T_{1/2\beta}$ (1.69 h) and Cl_B (0.13 L/kg·h) indicated a rapid distribution and elimination of cefquinome in black swans after IV administration. After IM injection, the corresponding PK parameters of $T_{1/2Ka}$, $T_{1/2Ke}$, T_{max}, C_{max}, and F were 0.12 h, 1.62 h, 0.39 h, 5.71 μg/mL and 74.2%, respectively. The MICs of cefquinome against black swans *E. coli* ranged from 0.03 to 8 μg/mL, with MIC_{50} and MIC_{90} of 0.06 and 0.5 μg/mL, respectively. The PK/PD cutoff of cefquinome against *E. coli* was determined to be 0.2 μg/mL. Monte Carlo simulation showed that the nominal dose regimen (2 mg/kg/24 h) could not achieve a satisfactory probability of target attainment (PTA) for $\%T_{MIC} \geq 50\%$, indicating a risk of treatment failure and the development of potential drug resistance.

Conclusions: The current daily dosage of cefquinome when divided into 12-h interval (1 mg/kg/12 h) may be effective for the treatment of *E. coli* infections with an MIC ≤0.5 μg/mL.

Keywords: Cefquinome, Black swans, Pharmacokinetics, Monte Carlo analysis

Background

The black swan (*Cygnus atratus*) is a large black-feathered waterbird which breeds mainly in New Zealand, Australia and adjacent coastal islands with nomadic migration patterns dependent on climatic conditions. As a popular ornamental bird, black swans have been introduced into numerous countries and have formed stable populations in zoological gardens [1]. In general, clinical bacterial infections caused by *Enterobacteriaceae* are common in waterfowl [2]. Additionally, *Escherichia coli, Salmonella* spp. and *Campylobacter* spp. infections can have serious detrimental on waterfowl populations and pose a potential threat to human public health as well. A recent study has reported the emergence of carbapenem and colistin-resistant *E.coli* isolates co-carrying bla_{NDM-5} and *mcr-1* genes in the fowls [3], indicating the possible spread and prevalence of such resistant strains through the food chain and migration of wild birds.

Cefquinome is the 4th generation cephalosporin antibiotic developed solely for veterinary use and has been approved for the treatment of many diseases including respiratory tract disease, foot rot in cattle, calf septicemia, metritis-mastitis-agalactia syndrome in sows, foal septicemia and respiratory diseases in horses [4]. Cefquinome is routinely used at a single dose of 2 mg/kg BW once-daily [5]. The advantages of cefquinome include broad-spectrum antimicrobial activity, highly stable

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against β-lactamases, enhanced potency and the ability to penetrate easily into Gram-negative bacterium [6]. Previous pharmacokinetic studies of cefquinome have been conducted in various species including pigs, cattle, beagle dogs, wild boars and ducks [7–11]. However, no relavant study regarding administration of cefquinome in wild animals was reported, and most previous studies were focused on the characteristics of drug disposition without regard to the appropriate dose regimen assessment for therapeutic use of cefquinome. Here we report, to the best of our knowledge, for the first time cefquinome PK properties and dose regimens assessment in wild birds.

In the present study, we present the PK profile and bioavailability of cefquinome in black swans. The MICs of cefquinome against 49 *E. coli* isolates from black swans were also determined. In addition, Monte Carlo analysis was performed to derive the corresponding daily dose regimens required to achieve the specific activity for various MIC breakpoints based on the determined PK parameters in black swans, the MIC distribution and the reported PD targets [12]. These results may provide fundamental data for the assessment of clinical efficacy of cefquinome and suggestions for a more rational dose regimen in black swans expected of having *E. coli* infections.

Methods

Animals and experimental design

Twelve healthy black swans weighting 5.14 ± 0.79 kg were divided equally into two groups. Each group randomly included three males and three females. Swans in each group received cefquinome (cefquinome sulfate injection, 40 mg/mL, Qilu Animal Health Products Co., Ltd., Jinan, China) at a single dose of 2 mg/kg/24 h BW by IV (brachial vein) or IM (chest muscle) injection. The black swans used in this study were kindly provided by the Lv-Yuan Rare Bird Farm (Shangdong, China). All birds were raised in accordance with the National Standards for Laboratory Animals of China (GB 14925–2010), allowed ad libitum access to water and antibacterial-free feedstuffs. The animal experimental protocol was approved by the Committee on the Ethics of animals of South China Sea Fisheries Research Institute and the Institutional Animal Care and Use Committee of Lv-Yuan Rare Bird Farm.

Sample collection and analysis

Blood (0.5 mL) was collected from the contralateral brachial vein with heparin sodium before and at 0.083, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 h following IV or IM administration. Plasma samples were then immediately isolated by centrifugation at 4000 rpm for 10 min and stored at −80 °C until further analysis.

A 0.2 mL aliquot of plasma sample was transferred into a capped centrifuge tube, and then mixed with 0.2 mL of acetonitrile. After vortexing (30 s) and centrifuging (12,000 rpm, 10 min), the supernatant was filtered through a 0.22 μm nylon syringe filter and collected into a sample vial for concentration determination. The plasma cefquinome concentrations were determined using a modified high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) method as our previously reported [9] (details are given in Additional file 1). The calibration standards in the linear range of 0.01–0.5 μg/mL were produced by working solutions spiked in blank plasma after extraction. All samples that had concentrations above 0.5 μg/mL were diluted proportionally with the control plasma prior to extraction with acetonitrile. The limit of detection (LOD) and quantification (LOQ) for cefquinome in plasma were set according to signal-to-noise (S/N) ratio of 3:1 and 10:1, respectively. The analytical method was validated by assessing extraction efficiency and inter- and intra-day reproducibility at drug concentrations of 0.01, 0.1 and 0.5 μg/mL.

Pharmacokinetic analysis

Pharmacokinetic parameters of cefquinome were estimated by a compartmental method using WinNonlin software (version 6.1, Pharsight, St. Louis, MO, USA). The best-fitting model required to describe cefquinome time-concentration curves for each swan was determined by application of the weighed residual sums of square and Akaike Information Criterion (AIC) methods [13]. The time-concentration of IV route was best fitted in WinNonlin program using a two-compartment model as presented in Model 7: Concentration (T) = $Ae^{-\alpha \cdot T} + Be^{-\beta \cdot T}$. The IM route was best fitted in WinNonlin program using a one-compartment model with the first-order absorption as presented in Model 3: Concentration (T) = $\frac{\text{Dose} \cdot \text{Ka}}{V_d\,(\text{Ka-Ke})}\, e^{-\text{Ke}\cdot T} - e^{-\text{Ka}\cdot T}$. For IV dosing, the distribution and elimination half-lives were estimated as $T_{1/2\alpha} = 0.693/\alpha$ and $T_{1/2\beta} = 0.693/\beta$, respectively. The half-lives of the first-order absorption and elimination after IM injection were correspondingly calculated as $T_{1/2Ka} = 0.693/Ka$ and $T_{1/2Ke} = 0.693/Ke$. The other PK parameters, peak plasma concentration (C_{max}), the time to C_{max} (T_{max}), the apparent steady-state volume of distribution (V_{ss}), total area under time-concentration curve (AUC) and total body clearance (Cl_B) were also calculated in WinNonlin software. The bioavailability (F%) was calculated according to the standard equations as follows [14]: $F = \frac{AUC_{IM}}{AUC_{IV}} \times 100\%$. All PK parameters were presented as mean ± SD values.

MIC determination

A total of 49 *E. coli* strains were obtained from more than 300 faecal swabs of black swans breeded in five different separated populations between 2014 and 2015. The MICs of cefquinome for these *E. coli* isolates were determined using the standard CLSI microdilution method [15]. The MICs for 50% and 90% of the isolates (MIC_{50} and MIC_{90}, respectively) were calculated accordingly.

Monte Carlo analysis and dose assessment

For β-lactam antibiotics acting by the time-dependent killing mechanisms, it is commonly recommended that the duration of time that drug levels exceed the MIC ($\%T_{MIC}$) should be at least 50% and possibly more than 80% of the dosing interval to ensure an appropriate bactericidal effect [12]. To further assess the recommended dose regimens of cefquinome in black swans against *E. coli*, a 10,000-subject Monte Carlo analysis was conducted using the Crystal Ball Professional software (version 7.2.2; Oracle Corporation), based on the current PK parameters, MIC distribution and the PD targets ($\%T_{MIC}$ > 50 or 80%).

The $\%T_{MIC}$ values after IM injection was calculated using the following equation: $\text{Concentration (t)} = \frac{\text{Dose} \cdot \text{Ka} \cdot \text{F}}{V_d\,(\text{Ka-Kel})} e^{-\text{Ke}\cdot t} - e^{-\text{Ka}\cdot t}$, where concentration is the MIC, V_d is the apparent volume of distribution, F is bioavailability, Ka is constant of absorption rate and Ke is constant of elimination rate for IM administration. All PK parameters were assumed to be normally distributed in the form of mean and standard deviation (Table 1). In order to obtain a unimodal distribution, the two isolates with a MIC of 8 μg/mL were consequently removed, and the $\log_2$-transformed MIC distribution of the other 47 isolates was submitted to the non-linear least squares regression and standard goodness-of-fit test. The probability of attaining the $\%T_{MIC}$ targets at the specific MICs was accordingly calculated. The PK/PD cutoff (CO_{PD}) is defined as the MIC, at which the PTA for $\%T_{MIC}$ target (50%) was equal to 90% under the current clinical recommended dose (2 mg/kg/24 h). For calculation of daily dose regimens, the MIC was defined as a single value ranging from 0.03 to 8 μg/mL. Scenarios were simulated separately for a single IM injection of cefquinome from 0 to 265 mg/kg with 24-h or 12-h intervals. The precise recipes of cefquinome required to achieve the specific activity ($\%T_{MIC}$ > 50 or 80%) against *E. coli* isolate at each MIC in black swans were also estimated based on PK data, Monte Carlo analysis and the equation mentioned above.

Results

Cefquinome concentration assay in swan plasma

The matrix-matched calibration curve was linear between 0.01 and 0.5 μg/mL, with a coefficient of determination (R^2) of 0.998. The LOQ and LOD were 0.01 and 0.005 μg/mL, respectively. Mean extraction recoveries from the five replicate assays were 88.9 ± 7.96%, 95.2 ± 7.20% and 98.3 ± 7.97% at spiked drug concentrations of 0.01, 0.1, and 0.5 μg/mL, respectively. The intraday coefficients of variation for replicate control samples (n = 5) within these concentration ranges varied from 1.52 to 5.69%, and the interday coefficients of variation ranged from 5.96 to 7.18%.

Table 1 Mean ± SD values of the pharmacokinetic parameters of cefquinome in black swans after IV and IM administration at a dose of 2 mg/kg BW

Parameter	Unit	IV	IM
Ka	1/h	—	5.64 ± 3.07
A	μg/mL	10.7 ± 4.97	—
α	1/h	2.27 ± 0.23	—
Kel	1/h	—	0.43 ± 0.03
B	μg/mL	4.20 ± 1.91	—
β	1/h	0.42 ± 0.09	—
V_{ss}	L/kg	0.32 ± 0.17	—
$T_{1/2Ka}$	h	—	0.12 ± 0.04
$T_{1/2\alpha}$	h	0.31 ± 0.03	—
$T_{1/2Ke}$	h	—	1.62 ± 0.11
$T_{1/2\beta}$	h	1.69 ± 0.85	—
T_{max}	h	—	0.39 ± 0.19
C_{max}	μg/mL	—	5.71 ± 1.43
AUC	μg·h/mL	16.5 ± 4.92	12.17 ± 4.32
Cl_B	L/kg·h	0.13 ± 0.04	—
F	%	—	74.2 ± 26.3

A, zero-time intercept of the distribution slope in the compartment model; B, zero-time inter of decline in plasma concentration of drug; α, distribution rate constant; β, elimination constant; Ke, constant of elimination rate; Ka, constant of absorption rate; $T_{1/2Kel}$, elimination half-life; $T_{1/2\alpha}$, the distribution half-life; $T_{1/2\beta}$, the half-life of elimination; $T_{1/2Ka}$, absorption half-life; V_{ss}, the apparent steady-state volume of distribution; Cl_B, total body clearance; AUC, total area under the concentration–time curve from zero to infinity; T_{max}, time to C_{max} from time zero; C_{max}, peak plasma concentration; F, bioavailability

Pharmacokinetics of cefquinome in plasma

No adverse effect or intolerance was observed during the entire experiment. The plasma time-concentration profiles are plotted in Fig. 1, and the corresponding PK parameters are summarized in Table 1. Cefquinome exhibited a biphasic decline and showed the best fit to a two-compartmental open model after IV dosing in black swans. The distribution half-life ($T_{1/2\alpha}$) and elimination half-life ($T_{1/2\beta}$) were 0.31 ± 0.03 h and 1.69 ± 0.85 h, respectively. After IM injection, the drug time-concentration data was best described by a one-compartment model with first-order absorption. Cefquinome was absorbed rapidly with an absorption

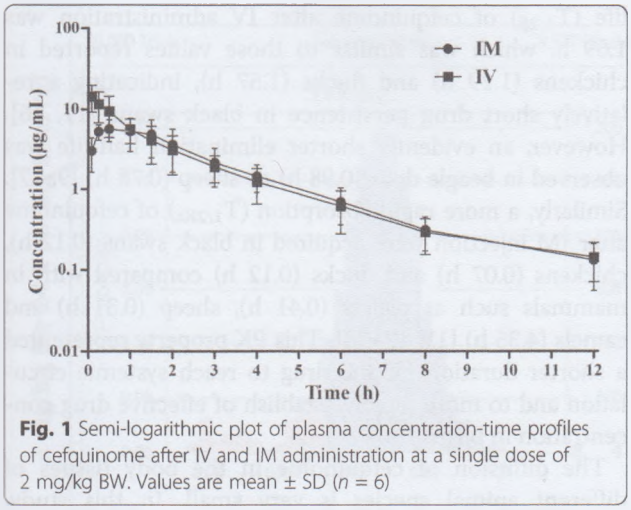

Fig. 1 Semi-logarithmic plot of plasma concentration-time profiles of cefquinome after IV and IM administration at a single dose of 2 mg/kg BW. Values are mean ± SD (n = 6)

half-life ($T_{1/2Ka}$) of 0.12 h. The peak concentration (C_{max}; 5.71 μg/mL) was achieved at 0.39 h, and the bioavailability was 74.2 ± 26.3% after IM injection. No drug was detected at 24 h after cefquinome administration.

MIC distribution

As shown in the primitive cefquinome MIC distribution in Fig. 2a, the MICs for cefquinome against 49 *E. coli* strains isolated from black swans were in the range of 0.03 to 8 μg/mL. The MIC_{50} and MIC_{90} were determined to be 0.063 and 0.5 μg/mL, respectively (Additional file 2: Table S1). The distribution percentage at each MIC (0.03, 0.06, 0.13, 0.25, 0.5, 1, 2 and 8 μg/mL) was 26.5%, 42.9%, 14.3%, 6.1%, 2.0%, 2.0%, 2.0% and 4.1%, respectively. In the fitted log_2-transformed MIC distribution of the 47 isolates, the best fit for the unimodal population was found (R^2 = 0.85) when presumed MIC distribution was defined as being between 0.03 and 2.0 μg/mL.

Monte Carlo analysis and dose assessment

The PTA values of cefquinome administered at single dose of 2 mg/kg/24 h against *E. coli* at each MIC were presented in Fig. 3. When MIC of *E. coli* was below 0.2 μg/mL, the PTA for achieving $\%T_{MIC}$ > 50% was as high as 90.2%. Therefore, the PK/PD cutoff of cefquinome against 49 *E. coli* isolates obtained in our study was 0.2 μg/mL. In addition, the calculated daily

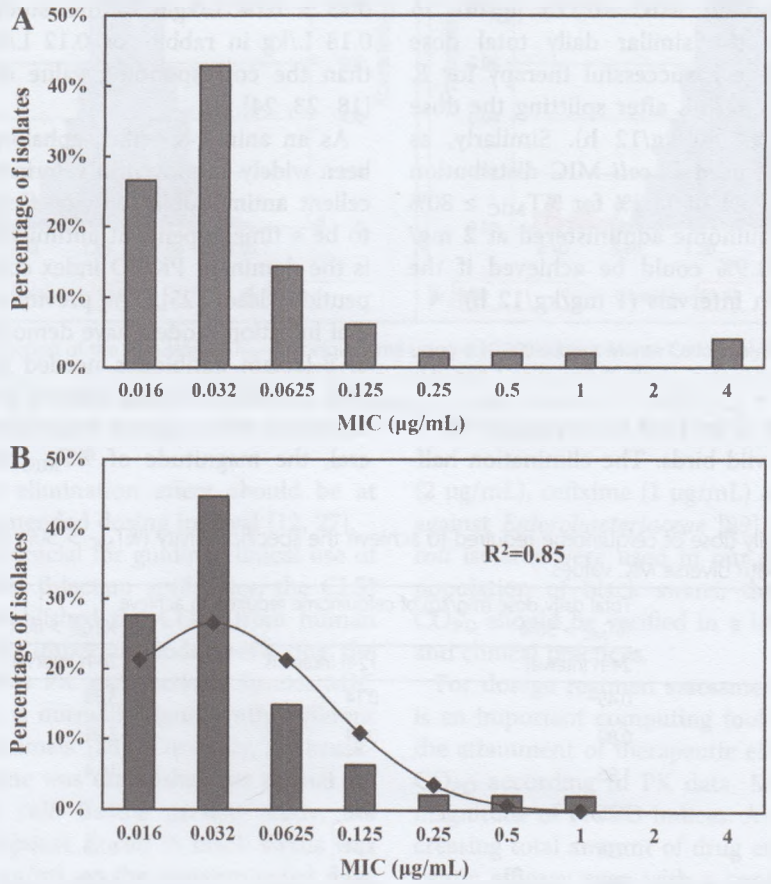

Fig. 2 The MIC distribution of cefquinome against *E. coli* isolates from black swans. **a** Primary MIC distribution of 49 *E. coli* isolates; (**b**) Fitted MIC distribution of the estimated 47 *E. coli* isolates after the goodness-of-fit tests and nonlinear least-squares regression. The *lines* indicate fitted theoretical normal distribution values

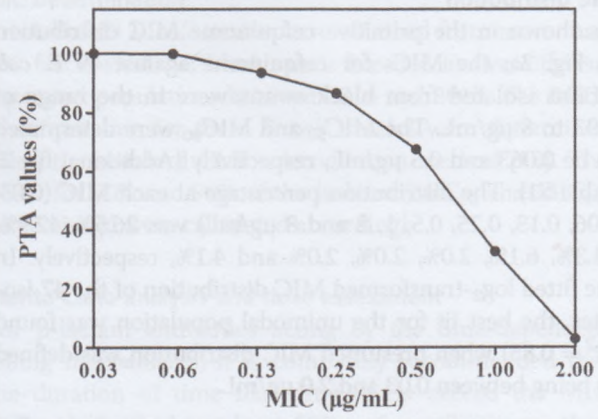

Fig. 3 The probability of target attainment (PTA) of cefquinome treated with the current clinical dose regimen (2 mg/kg/24 h) against *E. coli* at each MIC value

dosages of cefquinome required to achieve the specific activity ($\%T_{MIC}$ > 50% or 80%) against *E. coli* isolates with different MICs in black swans were summarized in Table 2. A simulated dose regimen (1.86 mg/kg/24 h) would be only therapeutically effective against *E. coli* with MIC ≤0.125 μg/mL in black swans. However, the similar daily total dose (1.94 mg/kg) may achieve a successful therapy for *E. coli* with a MIC of ≤0.5 μg/mL after splitting the dose into 12-h intervals (0.97 mg/kg/12 h). Similarly, as shown in Fig. 4, for the used *E. coli* MIC distribution in the present study, a PTA of 11.2% for $\%T_{MIC} \geq 80\%$ was acquired when cefquinome administered at 2 mg/kg once-daily, while 91.9% could be achieved if the same dose given at 12-h intervals (1 mg/kg/12 h).

Discussion

This study was the first of its kind to investigate PK of cefquinome applied in wild birds. The elimination half-life ($T_{1/2\beta}$) of cefquinome after IV administration was 1.69 h, which was similar to those values reported in chickens (1.29 h) and ducks (1.57 h), indicating a relatively short drug persistence in black swans [11, 16]. However, an evidently shorter elimination half-life was observed in beagle dogs (0.98 h) or sheep (0.78 h) [9, 17]. Similarly, a more rapid absorption ($T_{1/2Ka}$) of cefquinome after IM injection were acquired in black swans (0.12 h), chickens (0.07 h) and ducks (0.12 h) compared with in mammals such as piglets (0.41 h), sheep (0.31 h) and camels (4.35 h) [11, 17–20]. This PK property represented a shorter duration for the drug to reach systemic circulation and to more rapidly establish of effective drug concentration in birds.

The diffusion of cefquinome in the body tissues of different animal species is very small. In this study, barely 0.32 L/kg of V_{ss} was obtained in black swans similar to 0.25 L/kg in piglets and 0.19 L/kg in pigs [21, 22]. According to the CVMP report, an attenuated distribution of cefquinome to intracellular spaces was due to low fat solubility and that it acts as an organic acid with a low pKa of 2.51 or 2.91 [5]. In addition, total body clearance (Cl_B) in black swans was determined to be 0.13 ± 0.04 L/kg·h in this study, which is similar to 0.18 L/kg in rabbits or 0.12 L/kg in horse but lower than the corresponding value of 0.26 L/kg in piglets [18, 23, 24].

As an animal-specific cephalosporin, cefquinome has been widely employed in veterinary medicine due to excellent antimicrobial activity. Cefquinome is considered to be a time-dependent antimicrobial agent, and $\%T_{MIC}$ is the dominant PK/PD index correlated with the therapeutic efficacy [25]. The previous PK/PD studies in animal infection models have demonstrated that drug levels of β-lactam antibiotics needed to exceed the MIC for 36% to 40% of dosing interval to exert an in vivo bacteriostatic effect against *Enterobacteriaceae* [26]. In general, the magnitude of $\%T_{MIC}$ to ensure a significant

Table 2 Calculated total daily dose of cefquinome required to achieve the specific activity ($\%T_{MIC}$ > 50% or 80%) against *E. coli* isolates from black swans with diverse MIC values

MIC (μg/mL) for *E. coli* isolates	Total daily dose (mg/kg) of cefquinome required to achieve $\%T_{MIC}$ > 50%		$\%T_{MIC}$ > 80%	
	24-h interval	12-h intervals	24-h interval	12-h intervals
0.031	0.45	0.14	3.88	0.38
0.063	0.89	0.24	7.95	0.72
0.125	1.86	0.48	16.4	1.68
0.25	3.25	0.98	33.7	2.96
0.5	7.38	1.94	67.3	6.54
1	14.6	3.86	132.7	12.9
2	29.7	7.72	264.2	27.4
8	115.1	31.2	–	106.6

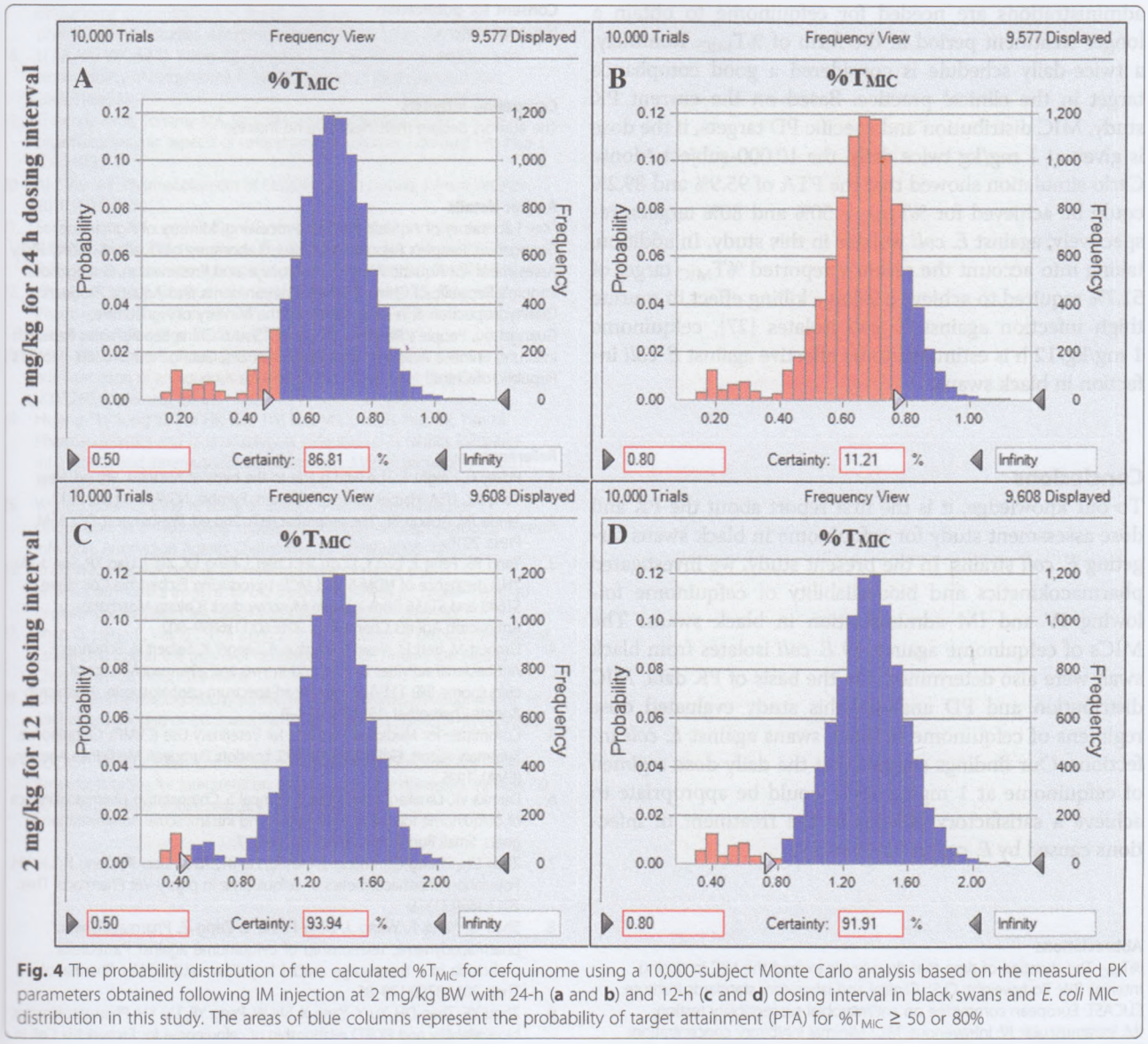

Fig. 4 The probability distribution of the calculated $\%T_{MIC}$ for cefquinome using a 10,000-subject Monte Carlo analysis based on the measured PK parameters obtained following IM injection at 2 mg/kg BW with 24-h (**a** and **b**) and 12-h (**c** and **d**) dosing interval in black swans and *E. coli* MIC distribution in this study. The areas of blue columns represent the probability of target attainment (PTA) for $\%T_{MIC} \geq 50$ or 80%

bactericidal or virtual elimination effect should be at least 50% of the recommended dosing interval [12, 27].

The PK/PD cutoff is crucial for guiding clinical use of antimicrobials. For most β-lactam antibiotics, the CLSI and EUCAST have established the CO_{PD} from human studies. However, as an important index reflecting the variations in host species PK and bacterial species MIC distribution, the CO_{PD} is normally significantly different between human and animals [28]. Currently, no breakpoint data of cefquinome was established for animal infections caused by *E. coli*. In the present study, the CO_{PD} of cefquinome against *E. coli* in black swans was determined to be 0.2 μg/mL at the recommended dose (2 mg/kg/24 h) based on Monte Carlo analysis, which was lower than the EUCAST clinical CO_{PD} values of cefotaxime (2 μg/mL), cefpodoxime (1 μg/mL), ceftriaxone (2 μg/mL), cefixime (1 μg/mL) and ceftibuten (1 μg/mL) against *Enterobacteriaceae* [29]. However, as only 49 *E. coli* isolates were used in our study due to the limited population of black swans, the relatively conservative CO_{PD} should be verified in a larger number of bacteria and clinical practices.

For dosage regimen assessment, Monte Carlo analysis is an important computing tool that is useful to predict the attainment of therapeutic efficacy and determine the CO_{PD} according to PK data, MIC distribution and the magnitude of PK/PD indices. As seen in Table 2, the increasing total amount of drug exerted little added therapeutic efficacy even with a considerably high simulated dose. However, after splitting into 12-h intervals, the identical daily dose could achieve a more satisfactory outcome than a single dosing. Therefore, more frequent

administrations are needed for cefquinome to obtain a longer treatment period in the form of $\%T_{MIC}$. Routinely, a twice-daily schedule is considered a good compliance target in the clinical practice. Based on the current PK study, MIC distribution and specific PD targets, if the dose is given at 1 mg/kg twice daily, the 10,000-subject Monte Carlo simulation showed that the PTA of 95.9% and 89.2% could be achieved for $\%T_{MIC} > 50\%$ and 80% targets, respectively, against *E. coli* isolates in this study. In addition, taking into account the recently reported $\%T_{MIC}$ target of 51.7% required to achieve a 2-$\log_{10}$ killing effect in murine thigh infection against *E. coli* isolates [27], cefquinome 1 mg/kg/12 h is estimated to be effective against *E. coli* infection in black swans.

Conclusions

To our knowledge, it is the first report about the PK and dose assessment study for cefquinome in black swans targeting *E. coli* strains. In the present study, we investigated pharmacokinetics and bioavailability of cefquinome following IV and IM administration in black swans. The MICs of cefquinome against 49 *E. coli* isolates from black swan were also determined. On the basis of PK data, MIC distribution and PD analysis, this study evaluated dose regimens of cefquinome in black swans against *E. coli* infections. Our findings suggest that the daily dose regimen of cefquinome at 1 mg/kg/12 h would be appropriate to achieve a satisfactory efficacy in the treatment of infections caused by *E. coli* in black swans.

Abbreviations

$\%T_{MIC}$: The duration of time that drug levels exceed the MIC in dosing interval; BW: Bodyweight; CLSI: Clinical and laboratory standards institute; EUCAST: European committee on antimicrobial susceptibility testing; IM: Intramuscular; IV: Intravenous; MIC: Minimal inhibitory concentration; PD: Pharmacodynamic; PK: Pharmacokinetic; PTA: Probability of target attainment

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Authors' contributions

LDL and DHZ designed and conducted the experiment. QW and XFW ran the simulations and analyzed the resulting data. DHZ drafted the manuscript. All authors read, revised and approved the final manuscript.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no interest.

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Clinical evaluation of a nutraceutical diet as an adjuvant to pharmacological treatment in dogs affected by Keratoconjunctivitis sicca

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Abstract

Background: Canine keratoconjunctivitis sicca (cKCS) is an inflammatory eye condition related to a deficiency in the tear aqueous fraction. Etiopathogenesis of such disease is substantially multifactorial, combining the individual genetic background with environmental factors that contribute to the process of immunological tolerance disruption and, as a consequence, to the emergence of autoimmunity disease. In this occurrence, it is of relevance the role of the physiological immune-dysregulation that results in immune-mediated processes at the basis of cKCS. Current therapies for this ocular disease rely on immunosuppressive treatments. Clinical response to treatment frequently varies from poor to good, depending on the clinical-pathological status of eyes at diagnosis and on individual response to therapy. In the light of the variability of clinical response to therapies, we evaluated the use of an anti-inflammatory/antioxidant nutraceutical diet with potential immune-modulating activity as a therapeutical adjuvant in cKCS pharmacological treatment. Such combination was administered to a cohort of dogs affected by cKCS in which the only immunosuppressive treatment resulted poorly responsive or ineffective in controlling the ocular symptoms.

Results: Fifty dogs of different breeds affected by immune-mediated cKCS were equally distributed and randomly assigned to receive either a standard diet (control, $n = 25$) or the nutraceutical diet (treatment group, $n = 25$) both combined with standard immunosuppressive therapy over a 60 days period. An overall significant improvement of all clinical parameters (tear production, conjunctival inflammation, corneal keratinization, corneal pigment density and mucus discharge) and the lack of food-related adverse reactions were observed in the treatment group ($p < 0.0001$).

Conclusions: Our results showed that the association of traditional immune-suppressive therapy with the antioxidant/anti-inflammatory properties of the nutraceutical diet resulted in a significant amelioration of clinical signs and symptoms in cKCS. The beneficial effects, likely due to the presence of supplemented nutraceuticals in the diet, appeared to specifically reduce the immune-mediated ocular symptoms in those cKCS-affected dogs that were poorly responsive or unresponsive to classical immunosuppressive drugs. These data suggest that metabolic changes could affect the immune response orchestration in a model of immune-mediated ocular disease, as represented by cKCS.

Keywords: Antioxidant and anti-inflammatory diet, Immune-mediated ocular disease, Keratoconjunctivitis sicca, Nutraceutical diet

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Background

Keratoconjunctivitis sicca, also defined as "dry eye disease" or Sjögren's syndrome in human [1], is a tear film disorder which causes inter-palpebral ocular surface damage and is associated with ocular discomfort [2, 3] both in humans and dogs [4, 5]. Canine keratoconjunctivitis sicca (cKCS) is an inflammatory eye condition which affects both cornea and conjunctiva and that is related to a deficiency in tear aqueous fraction [6]. The prevalence of such disease is estimated in about 4% when considering Schirmer test I (STT) values < 10 mm/min [7] reaching the 64% in male crossbred dogs between six to nine years of age [8]. Moreover, it is often an under-recognized and/or a sub-clinical condition [9] which, in some breeds, is preceded by an immune-mediated destruction of lachrymal glands [10, 11].

In this regard, the immune-mediated mechanisms of cKCS or of human, like the Sjögren's syndrome [1] induction are not clearly defined. Etiopathogenesis of such disease is substantially multifactorial, combining the individual genetic background with environmental factors that contribute to the process of immunological tolerance disruption and, as consequence, to the autoimmunity processes [12–14]. It is of relevance the role of the physiologic immune-dysregulation that results in the autoimmune process of cKCS and Sjögren's syndrome [12–15]. Notably, the T and B cell infiltration, the recruitment of dendritic cells, the up regulation of those molecules fostering the antigen presentation as well as the increased secretion of pro-inflammatory cytokines, such as interferon (IFN)-γ [16], in ocular tissues have been demonstrated to contribute to the inflammatory alterations of the lachrymal gland [17–19]. This process usually results in mucopurulent-like eye discharge, conjunctival hyperemia, keratitis, corneal pigmentation, neovascularization and blepharospasm in cKCS [20, 21].

Current therapies for this ocular disease rely on immune-suppressive treatments, represented by Cyclosporine A [22], glucorticoid [21], tacrolimus [23] and artificial tears in order to recover an adequate eye's lubrication [24]. Nevertheless, recognized complementary or alternative therapeutical approaches are represented by the cholinergic agents (pilocarpine) [25] and the surgical treatments (punctal occlusion, tarsorrhaphy, conjunctival flaps, contact lenses, superficial keratectomy, as well as parotid duct transposition) [26]. Clinical response to treatment frequently varies from poor to good, depending on the clinical-pathological status of eyes at diagnosis and on individual response to therapy [13]. Among other causes of cKCS traumas [27], congenital causes [28], distemper [29], radiation therapy [30, 31], neurological deficit [32], diabetes mellitus [33] and uncorrected prolapse of the nictitans gland [34] are of note. Intriguingly, majority of these aspects could correlate and contribute to both the determinism and exacerbation of inflammatory condition in ocular tissue.

In the light of the variability of clinical response to classical therapies, it could be useful the use of therapeutical adjuvants in cKCS management to improve the response to pharmacological treatment. Thus, we evaluated a combined therapeutical approach based on the classical drug administration and the use of an anti-inflammatory/antioxidant diet with potential immune-modulating activity. Such combination was administered to a cohort of cKCS dogs in which the only immune-suppressive treatment resulted poorly responsive or ineffective to control the ocular symptoms.

The nutraceutical diet used in this clinical evaluation consisted in a commercial mixed formula based on fish proteins, rice carbohydrates (whose carbohydrates percentage ranges from 75 up to 80, starch 65 to 70% with a beta-glucans quote of less than 0.1%), *Cucumis melo*, *Ascophyllum nodosum*, Astaxanthin (from *Hematococcus pluvialis*), *Aloe vera*, *Carica papaya*, *Punica granatum*, *Camellia sinensis*, *Polygonum cuspidatum*, *Curcuma longa*, *Piper nigrum*, zinc and a Omega3/6 ratio of 1:0.8), which already provided significant immunomodulating results, decreasing type 1 helper T lymphocyte (Th1) cells and increasing T regulatory (Treg) cells, in dogs affected by *Leishmania infantum* [35].

Cucumis melo (melon) shares some anti-oxidant and anti-inflammatory properties that involve the superoxide/peroxynitrite clearance and the modulation of macrophagal interleukin-10 production [36], while the immune-modulating activity is exerted by the induction of type 1 helper T lymphocyte (Th1) polarization [37].

The *Ascophyllum nodosum* activity is related to the presence of a sulfated-polysaccharide, ascophyllan, able to induce nitric oxide, tumor necrosis factor (TNF)-α and granulocyte colony-stimulating factor (GM-CSF) secretion in macrophages [38]. Astaxanthin, an orange-pinkish carotenoid, is known to act on polyunsaturated fatty acids oxidation [39], inflammatory responses modulation, and to promote eye's health in humans and animals [40]. This carotenoid induces lymphoblastogenesis and lymphocyte cytotoxicity in mice [41] as well as T-cell and B lymphocyte proliferation and natural killer cytotoxicity in humans [42]. Reduced production of Interleukin (IL)-1β, IL-6, TNF-α and IL-10 has been observed in vitro after the addition of *Aloe vera* (aloe) extracts to the culture of corneal cells [43]. The anti-inflammatory effect of *Carica papaya* (papaya) is related to an increase of regulatory T cells and a reduction of IFN-γ^+ $CD4^+$ T cells [44]. Reduction of IL-2 and IL-4 and enhancement of IL-12, interferon (IFN)-γ and TNF-α have been observed in blood mononuclear cells [45]. The seed oil and juice of *Punica granatum* (pomegranate) contains some flavonoids and anthocyanidins (delphinidin, cyaniding and pelargonidin)

with an antioxidant activity greater than green tea extract [46, 47]. Its antioxidant action is related to free radical scavenging by anthocyanidins [46] and to metal ions chelation [48]. A protective effects of *Punica granatum* on cardiovascular system has been correlated to angiotensin converting enzyme inhibition, blood pressure decrease [49] and endothelial nitric oxide syntase production [50]. *Punica granatum* also has been shown to inhibit cyclooxygenase, lipooxygenase [51] and IL-1β, modulate matrix metallo-proteinases in osteoarthritis, prevent collagen degradation [52], inhibit the p38-mitogen-activated protein kinase pathway and nuclear factor kappa (NF-kB) light-chain-enhancer in B cells [53, 54], and decrease malondialdehyde, TNF-α, IL-1β and IL-6 [55, 56].

The antioxidant effects of *Camellia sinensis* (green tea) are exerted through radicals scavenging and lipid-peroxidation inhibition [57] by flavonoids (catechin, epicatechin, epigallocatechin and gallate esters) [58]. In this context, epigallocatechin-3-gallate is known to inhibit UVB-mediated erythema, hydrogen peroxide production, leukocyte infiltration [59], matrix metallo-proteinases [60, 61], neutrophil chemotaxis [62], degradation of cartilage [63], TNF-α expression [64], neutrophil-mediated angiogenesis [62] and reduce the cyclooxygenase-2 and neutral endopeptidase activity [65]. *Polygonum cuspidatum* (japanese knotweed), a natural source of resveratrol, is endowed with anti-inflammatory and antioxidant activities [66, 67]. Resveratrol has been shown to directly act on TANK-binding kinase 1, an integral component in chronic inflammatory diseases [68], and on arteries by activating the nitric oxide/soluble guanylyl cyclase pathway [69]. Its anti-inflammatory effect is supposed to be regulated by estrogen receptor-α [70]. Moreover, certain resveratrol dimers (parthenocissin A, quadrangularin A and pallidol) exert free radical quenching and, selectively, single oxygen scavenging activity [71]. *Curcuma longa* (curcuma) induces powerful free radicals scavenging effect and anti-inflammatory activity [72, 73]. Curcumin, one of the constituents of such plant, reduces leukocyte adhesion and superoxide production, stimulates spontaneous apoptosis and inhibits IL-8 [74].

Moreover, a down regulation of Th1 cytokine response and of macrophagal nitric oxide production has also been observed [75]. The anti-inflammatory effect of curcumin involves the inhibition of NF-kB in activated B cells and the down-regulation of TNF-α and IL-6 [73] as well as the up-regulation of nuclear factor erythroid 2 activity [76], whose downstream proteins are involved in the protection mechanisms against oxidative stress [77]. *Piper nigrum* (pepper) commonly used in the treatment of flu, cold, rheumatism, pain, muscular aches, chills, exhaustion, fevers, is used as a useful nerve tonic also able to increase blood circulation and saliva production as well as to stimulate appetite and peristalsis [78]. It is also known to enhance the effectiveness and bioavailability of curcumin [79] by acting on membrane lipid dynamics in reason of the apolar nature of piperine, the main bioactive compound of *Piper nigrum*. Piperine has been shown to promote conformational changes of intestine enzymes [80] and significantly inhibit the expression of major histocompatibility complex class II, CD40 and CD86 in bone-marrow-derived dendritic cells as well as the production of TNF-α and IL-12 by the same cells [81]. In addition, piperine was proven to attenuate inflammatory processes by partially acting on pituitary adrenal axis [82], reduce high-fat diet-induced oxidative stress [83, 84] and enhance pancreatic activity [85]. The deficiency of zinc affects both innate and adaptive immunity [86]. This element is crucial for the balance between the different T-cell subsets and its deficiency was shown to decrease the production of Th1 cytokines (IFN-γ, IL-2 and TNF-α), whereas the Th2 response (IL-4, IL-6 and IL-10) is affected in a lesser extent [87]. While acute zinc deficiency seems to correlate with the decrease in innate and adaptive immunity, its chronic deficiency is known to increase pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) production influencing the outcome of several inflammatory diseases [88].

An optimal balance of the omega Omega 3/6 fatty acids ratio represents a fundamental requirement for tissue homeostasis recovering during inflammatory responses. The polyunsaturated fatty acids, usually found in fish oil (i.e., eicosapentaenoic acid and docosahexaenoic acids), are known to decrease proinflammatory cytokine production and to inhibit natural killer cell activity [89]. The gamma-linolenic acid has been demonstrated to exert an anti-inflammatory activity by suppressing IL-1β and TNF-α secretion by monocytes [90]. Additionally, eicosapentaenoic supplementation might foster the anti-inflammatory activity of gamma-linolenic acid by decreasing the synthesis of arachidonic acid and prostaglandin E2 [91].

Here, we evaluated the use of a commercially available nutraceutical diet as a therapeutical adjuvant in cKCS-affected dogs that were unresponsive to standard pharmacological therapies.

Methods

Experimental design, dogs and diets

This evaluation was designed as a randomized, placebo-controlled clinical one. Fifty client-owned dogs (19 females and 31 males) aged 6.5 ± 0.7 years [mean ± Standard Error of Mean] of different breeds (one poodle, two dachshund long hair, four dachshund smooth coat, four west highland white terrier, two yorkshire terrier, four maltese, one bulldog, two chinese crested dog, two chinese pug, eight shih tzu, four german shepherd, 10 mixed breed,

two chow chow, two cocker, two english setter) were enrolled in this evaluation. All dogs were previously evaluated by an Italian Animal Health Foundation certified panelist (Dr D. Giretto) to confirm the diagnosis of immune-mediated KCS. Inclusion criteria were the presence of blepharospasm, conjunctival inflammation, corneal keratinization, corneal pigmentation density, neovascularization, mucus discharge and a STT value < 10 mm/min. Exclusion criteria were the presence of correlated systemic diseases, neurological disease, traumatic and toxic keratoconjunctivitis, in order to better evaluate the clinical response to the immune-mediated cKCS, or general symptoms of intolerance/allergy to ingredients of the nutraceutical diet tested in this clinical evaluation. Moreover subjects affected by neurological cKCS were excluded.

Dogs were randomly and equally divided into two groups: 25 dogs fed a standard diet (SD group), as control group, and 25 fed an antioxidant/anti-inflammatory nutraceutical diet (ND group), as experimental group. Male and female dogs were equally represented in both groups. Regardless the type of diet, all dogs were treated over a 60 days period as follows: [0,03% Tacrolimus collyrium diluted into a benzalkonium chloride and methyl cellulose solution (Lacrimart, Fedel Farma S.r.l., Chieti, Italy) BID and 0,2% Hyalistil eye drops (artificial tears, S.I.F.I. S.p.A. Aci S. Antonio, Catania, Italy) five times a day] ([http://eng.forza10.com/immuno-active-755-2.html]).

The recommendations of the ARRIVE guidelines in animal research were consulted and considered [92].

In Table 1, we reported the background data of the dogs belonging to both groups along with their scores before starting the evaluation.

Both diets completely fulfil the recommendations for proteins, carbohydrates and fats in order to obtain a complete food for a daily ration in dog, as reported in Nutritional Guidelines for complete and complementary pet food for cats and dogs by The European Pet Food Industry Federation. Foods were in the form of kibbles industrially produced with extrusion technique. ND and SD foods reported similar analytical composition in nutrients (24% of crude protein, 12% of crude oils and fats, 3.7%, of crude fiber 5% of crude ash, 9% of moisture). Both diets had analogue recipes and included the same macro and micro nutrients including vitamins, trace elements and minerals. The two foods differed mainly from the presence of botanicals in ND food. ND food was composed by two mixed components: kibbles, included in the ideal percentage of 93-94% in weight, and cold-pressed tablets at the 6-7% in weight of complete food (European patent n. EP 2526781). Tablets were composed by 60-80% of protein hydrolyzed (fish and vegetable ones), 20-40% of minerals used as glidants and were added by therapeutical substances (*Ascophyllum nodosum, Cucumis melo, Carica papaya, Aloe vera, Astaxanthin from Haematococcus pluvialis, Curcuma longa, Camellia sinensis, Punica granatum, Piper nigrum, Poligonum spp, Echinacea purpurea, Grifola frondosa, Glycine max*, Omega 3 and Omega 6 unsaturated fatty acids from fish, as 1.60% and 1.25% of oil respectively).

The pet food used in SD group did not contain the above-mentioned active substances.

ND and SD dietary administration were administrated following a daily table recommendation (Table 2) and carefully adjusted during the trial to provide similar caloric animal food intake and to satisfy the nutritional requirement of adult dogs. In order to avoid any deficiency, the energy value of both complete food was calculated using the expression suggested by Nutritional Guidelines for Complete and Complementary Pet Food for Cats and Dogs and Nutrient requirements of dogs and cats, National research council of the National academies, (% crude protein x 3.5 + % crude fat x 8.5 + % NFE (Nitrogen-free extract) x 3.5). The correct dosage was calculated using another expression 110 kcal ME*kg $bw^{0.75}$ (Nutritional Guidelines for Complete and Complementary Pet Food for Cats and Dogs and Nutrient requirements of dogs and cats, National research council of the National accademies). The constant 110 is referred to the energy requested by a dog with normal physical activity. At the enrollment, each animal was weighed and the suggested daily ratio calculated. The Veterinarians clearly informed the owners about the correct dosage to be provided. Moreover the average of daily administered botanicals was calculated considering the ratio given to the dogs, related to the amount declared by the manufacturer. Table 3 highlights the average amount, in terms of mg/kg, of botanicals estimated according to the mean weight.

Ophthalmologic examination

Each dog was evaluated on day 0,15, 30, and 60 of the evaluation by an independent observer (SD, DG, CM).

Table 1 Background data of enrolled dogs

Group	Mean age (years ± SEM)	Mean weight (Kg ± SEM)	STT value (mm ± SEM)	Corneal pigment density score (0-3 ± SEM)	Conjunctival inflammation score (0-3 ± SEM)	Mucus discharge score (0-3 ± SEM)	Corneal keratinization score (0-2 ± SEM)
Control	6.03 ± 0.15	13.04 ± 1.12	4.3 ± 0.5	1.0 ± 0.1	2.1 ± 0.1	1.7 ± 0.1	1.5 ± 0.1
Treatment	6.1 ± 0.17	12.01 ± 1.17	4.7 ± 0.4	0.9 ± 0.1	2.1 ± 0.1	1.8 ± 0.1	1.5 ± 0.1

Table 2 Daily table recommendation for diet

Weight (Kg)	Diet amount per day (g)
1 – 10	30 – 180
11 – 20	190 – 300
21 – 35	310 – 455
36 – 50	465 – 595

Each dog underwent a complete ophthalmological examination by three board-certified veterinary ophthalmologists (Dr. M.C. Muscolo and Dr. S. De Stefanis are board-certified by the D'Ophtalmologie ENV Alfor; Dr. D. Giretto is board-certified by Certificat d'Etudes Superieur en Ophtalmologie ENV Toulouse and is an Italian Animal Health Foundation board member).

Ophthalmic examinations included, slit-lamp biomicroscopy (Kowa Optimed Inc SL-14 Slit Lamp, Kowa Optimed, Europe Ltd, Berkshire, UK), funduscopic examination (Heine Omega 180 Binocular Indirect Ophthalmoscope, HEINE Optotechnik, Herrsching, Germany), applanation tonometry (Tono-Pen® Vet, Reichert Technologies, Depew, NY, USA) preceded by an ocular application of oxybuprocaine hydrochloride 0.4% (Novesina Novartis Farma S.p.A, Origgio (VA), Italy) in order to reduce the nuisanceand fluorescein dye staining (fluorescein 0.5% collyre unidose TVM, Laboratoires TVM, Lempdes, France) along with 0.9% physiologic rinsing solution (Eurospital S.p.A., Trieste, Italy).

Both eyes of each dog were photographed at each visit in the afternoon (3–6 pm) and clinical signs, such as corneal pigment density and corneal keratinization, were graded according to the scores proposed by Hendrix et al. [93], whereas conjunctival inflammation and mucus discharge were graded according to the scores proposed by Moore et al. [94].

- corneal pigment density (0-3): 0 = no pigment, 1 = iris easily visualized through the pigment, 2 = iris partially visualized through the pigment, 3 = iris not visible through the pigment);
- conjunctival inflammation (0-3): 0 = normal conjunctiva; 1 = mild hyperemia without chemosis; 2 = moderate hyperemia with mild chemosis; 3 = intense hyperemia with moderate to severe chemosis;
- mucus discharge (0 – 3): 0 = no visible mucus or clear mucus thread; 1 = scattered non-adherent mucopurulent strands; 2 = moderate adherent mucopurulent strands covering up to 25% of the cornea; and 3 = diffuse extensive adherent mucopurulent discharge covering 25% to 50% of the cornea;
- corneal keratinization (0-2): 0 = none, 1 = mild opacity, 2 = moderate opacity.

Enrolled dogs were treated by their owners at home by applying the pharmacological treatment as previously described and the diet administration approximately every 12 h.

Schirmer tear test

Schirmer tear test-1 (STT-1) is a routine examination which is performed by placing a standard test strip (Schirmer-Plus®, Gecis Ecoparc, Domaine de Villemorant, France) within the ventral conjunctival sac of each dog for 60 s. Tear production is then recorded in mm/min for each eye. STT-1 was performed on 100 eyes of dogs of several breeds.

Statistical analysis

Data were analyzed using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). All data are presented as the means ± standard error of the mean and were first checked for normality using the D'Agostino-Pearson normality test. Differences in Schirmer test, conjunctival inflammation, corneal keratinization, corneal pigmentation density and mucus discharge score between

Table 3 Average substances administer to dog depending on body weight (considering medium body weight)

Nutraceutical substances	Amount per kg of complete food		Dog weight 10 kg	11 kg	12 kg	13 kg
Ascophyllum nodosum	40000	mg/kg	7200	7600	8200	8600
Cucumis melo	300	mg/kg	54	57	61,5	64,5
Carica papaya	135	mg/kg	24,3	25,65	27,675	29,025
Aloe vera	135	mg/kg	24,3	25,65	27,675	29,025
Haematococcus pluvialis (astaxanthin)	49	mg/kg	8,82	9,31	10,045	10,535
Resveratrol (Poligonum Cuspidatum)	7	mg/kg	1,26	1,33	1,435	1,505
Zinc sulphate monohydrate	137	mg/kg	24,66	26,03	28,085	29,455
Curcuma longa	102	mg/kg	18,36	19,38	20,91	21,93
Camellia sinensis	70	mg/kg	12,6	13,3	14,35	15,05
Punica granatum	70	mg/kg	12,6	13,3	14,35	15,05
Piper nigrum	30	mg/kg	5,4	5,7	6,15	6,45

the two treatments at the end of treatment versus baseline for each eye were blindly analyzed by ADC using a two-way analysis of variance (ANOVA) followed by Sidak's multiple comparisons test. Conjunctival inflammation, corneal keratinization, corneal pigmentation density and mucus discharge score between the two treatments at the end of treatment versus baseline for each eye were analyzed using a paired *t*-test. Veterinary ophthalmologists were not involved in the statistical analysis of the data.

Results and Discussion

Clinical evaluation of eyes in ND and SD group

Fifty dogs were enrolled in the trial: 25 dogs received the pharmacological treatment and a standard diet (SD Group), while 25 dogs received the pharmacological treatment plus an antioxidant/anti-inflammatory nutraceutical diet (ND Group).

An overall amount of 100 eyes was considered according to literature suggestions [95–97]. All dogs completed the 60-day evaluation period.

The overall improvement of eye's condition in two representative dogs of ND group at the day 0 of the trial (Fig. 1a, c) and at the end of the 60-days evaluation (Fig. 1b, d) is shown. In particular, our results highlight the clinical amelioration occurred in ND group (Fig. 1b, d) in terms of blepharospasm, ocular hyperemia, periocular swelling and ocular discharge that is strongly dependent on nutraceuticals administration since no effects were evident in SD group (Fig. 1e, h). In this regard, the comparative evaluation between the day 0 (Fig. 1e, g) and the end of 60-days (Fig. 1f, h) in two representative dogs of SD group showed none significant clinical amelioration. Indeed, blepharospasm, ocular hyperemia, periocular swelling and ocular discharge were still evident or, at least, poorly improved.

These results strongly pointed to a specific effect of nutraceuticals in inducing anti-inflammatory and immunemodulating outcomes in eyes of dogs belonging to ND group. Notably, the standard pharmacological treatment appeared to be substantially ineffective since no amelioration has been observed in dogs belonging to SD group. Therefore, the effect of nutraceuticals could be considered as highly fostering the clinical improvement during the pharmacological treatment in cKCS.

The eye's scores amelioration in cKCS dogs treated with ND

Figure 2 shows the eye's score intensity trend of each symptom of dogs belonging to SD and ND group.

Dogs conjunctival inflammation score significantly decreased from a baseline of 2.1 ± 0.1 to 0.6 ± 0.1 in the ND group, while no significant variation (from a score of 2.1 ± 0.1 to 1.9 ± 0.1) appeared in SD group (Fig. 2a–b).

In addition, corneal keratinization score resulted significantly decreased in ND group (from 1.5 ± 0.1 to 0.2 ± 0.1) and not in SD group (from 1.5 ± 0.1 to 1.4 ± 0.1)

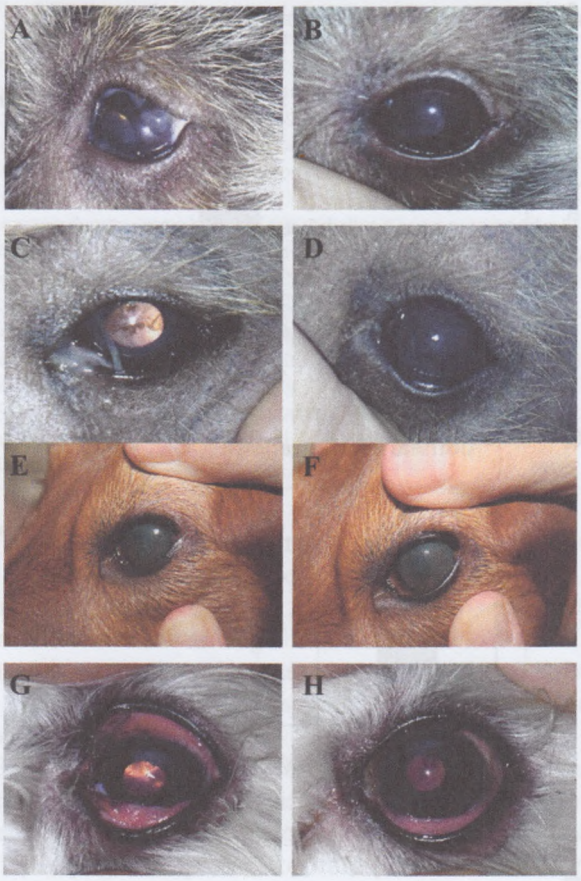

Fig. 1 Eyes improvement after 60-days treatment with 0,03% Tacrolimus collyrium BID and 0,2% Hyalistil eyewash plus the nutraceutical diet in ND group and with with 0,03% Tacrolimus collyrium BID and 0,2% Hyalistil eyewash plus the standard diet in SD group. **a–c** eye before treatment plus nutraceutical diet (time = 0), **b–d** eye at the end of treatment plus nutraceutical diet (time = 60). **e–g** eye before treatment plus standard diet (time = 0), **f–h** eye at the end of treatment plus standard diet (time = 60)

(Fig. 2c–d). Finally, corneal pigment density and mucus discharge resulted significantly decreased only in ND group, while no effects were evident in SD group. More in details, corneal pigment density scores decreased from a baseline value of 0.9 ± 0.1 to 0.2 ± 0.1 whereas mucus discharge scores decreased from 1.8 ± 0.1 to 0.3 ± 0.1 (Fig. 2e–h).

These results clearly suggest the role for ND in inducing the amelioration of eye's score testing in cKCS and that this occurrence appears independent on pharmacological treatment since drugs alone appeared ineffective, as evident in SD group.

As to STT-1 values, a significant increase was observed from a baseline value from 4.7 ± 0.4 mm to 10.7 ± 0.6 mm after the 60-days of treatment only in the dogs of ND group, while no significant improvement (STT-1 values from 4.3 ± 0.5 mm to 5.1 ± 0.5 mm) was evident in the dogs of SD Group at the end of the trial (Fig. 2i–l).

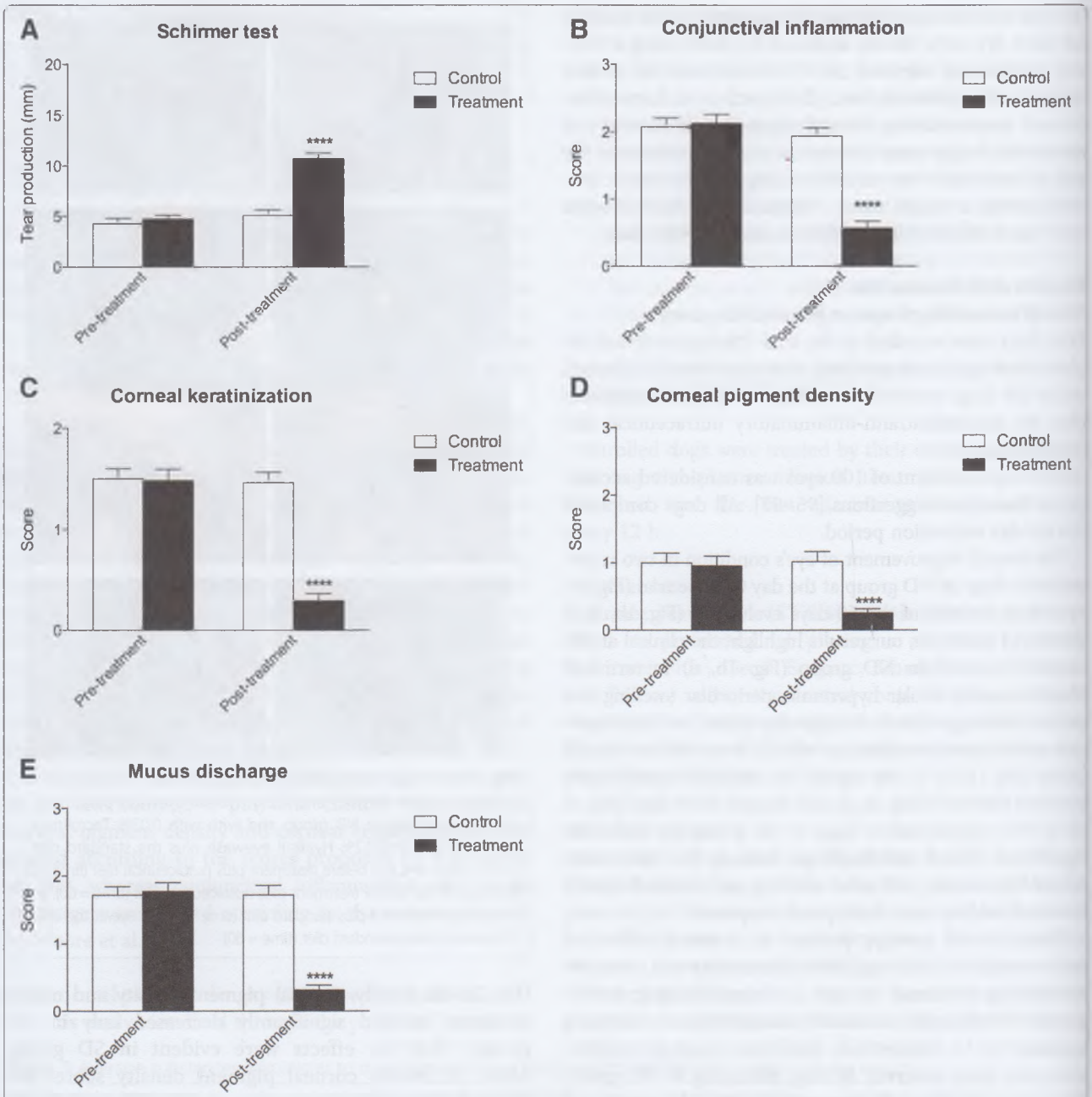

Fig. 2 **a** mean tear production (STT) in mm/min before and after 60 days treatment for ND and SD group, STT values resulted significantly increased (****$P < 0.0001$) in ND group at the end of treatment, **b** Mean conjunctival inflammation scores before and after 60 days treatment for ND and SD group, a significant decrease (****$P < 0.0001$) was observed in ND group at the end of the treatment; **c** mean corneal keratinization scores before and after 60 days treatment for ND and SD group, a significant decrease (****$P < 0.0001$) was observed in ND group at the end of the treatment; **d** mean corneal pigment density scores before and after 60 days treatment for ND and SD group, a significant decrease (****$P < 0.0001$) was observed in ND group at the end of the treatment; **e** mean mucus discharge scores before and after 60 days treatment for ND and SD group, a significant decrease (****$P < 0.0001$) was observed in ND group at the end of the treatment

These results evidenced the effectiveness of ND in increase the tear film in our cohort of sick dogs. It is reasonable that the anti-inflammatory effects of nutraceuticals could contribute to restore the physiological eye's tear production in cKCS.

The relapse/regression of cKCS symptoms in dependence of ND administration

After the 60 days of evaluation, dogs belonging to ND group interrupted the diet supplementation for 30 days, while continuing the pharmacological treatment.

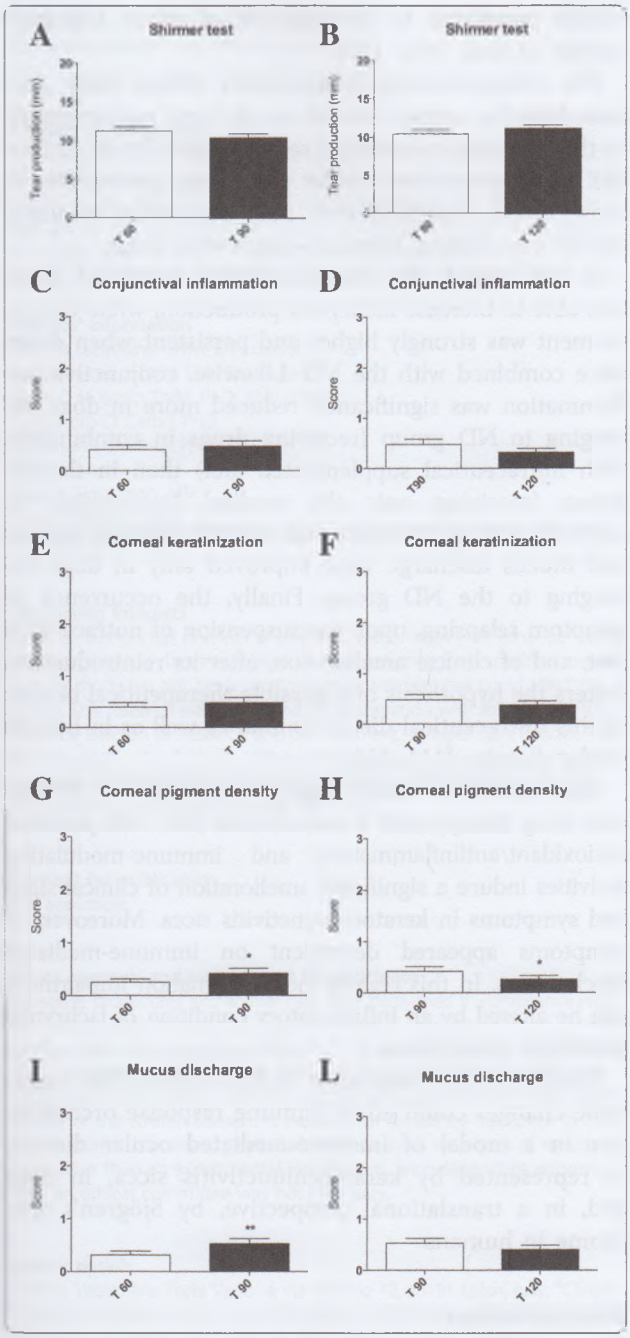

Fig. 3 Graphical schematization of clinical symptoms score trends after 30 days since treatment suspension and after 30 days since treatment resumption. **a** Mean conjunctival inflammation scores before (T 60) and after 30 days nutraceutical diet suspension (T 90) for ND group and (**b**) after 30 days since nutraceutical diet resumption (T 120); **c** mean corneal keratinization scores before (T 60) and after 30 days nutraceutical diet suspension (T 90) for ND group and (**d**) after 30 days since nutraceutical diet resumption (T 120); **e** mean corneal pigment density scores before (T 60) and after 30 days nutraceutical diet suspension (T 90) for ND group and (**f**) after 30 days since nutraceutical diet resumption (T 120), scores resulted significantly increased (*$P < 0.05$); **g** mean mucus discharge scores before (T 60) and after 30 days nutraceutical diet suspension (T 90) for ND group and (**h**) after 30 days since nutraceutical diet resumption (T 120), scores resulted significantly increased (**$P < 0.01$); **i** mean tear production (STT) in mm/min before (T 60) and after 30 days nutraceutical diet suspension (T 90) for ND group and (**l**) after 30 days since nutraceutical diet resumption (T 120)

It is worth noting that a rapid and intensive relapse of symptoms was observed after 15 days since ND suspension. All dogs were newly supplemented with the ND while continuing the pharmacological therapy for another 30 days. Intriguingly, an overall regression of symptoms was again observed after the reintroduction of ND (Fig. 3).

This occurrence clearly highlighted the specific effects of nutraceuticals as useful adjuvant in the treatment of cKCS-affected dogs, particularly for those animals poorly responsive or unresponsive to standard pharmacological therapy.

Conclusions

To the best of our knowledge, this clinical evaluation represents first study that proposed the use of a specific antioxidant/anti-inflammatory ND as an optimal combination of ingredients with synergistic effects able to potentially exert an immune-modulating activity in combination with standard pharmacological treatments in cKCS.

The nutraceutical approach appears to significantly increase the eye's tear production and to clinically ameliorate the conjunctival inflammation status as well as the corneal keratinization, corneal pigment density and mucus discharge in chronic cKCS dogs poorly responsive or unresponsive to immune-suppressive therapy.

The increased STT level in response to the proposed ND was in agreement with previously reported response to topical CsA and Tacrolimus [23, 98, 99]. Although we are unaware of the possible action mechanism of all ingredients, in particular for the phytotherapic extracts, we hypothesize that these substances and raw materials of the ND may exert a synergic action in the T-cell activation, possibly by preventing inflammatory gene transcription (IL-2, IL-3, IL-4, IFN- γ, TNF-α, GM-CSF, c-myc) [16, 100, 101].

Based on a possible mimicking action mechanism of all active substances with respect to CsA, we also hypothesized a reduced secretion of TNF-α by T cells. In this regard, TNF-α is known to increase mucin secretion from respiratory epithelial cells, thus it could possibly influencing the mucus production, corneal keratinization and conjunctival inflammation status [102, 103]. However, as observed by Hendrix et al. an overall significant improvement of clinical signs was not observed over time [93].

Intriguingly, our results seem to support the use of an anti-inflammatory/immune-modulating ND as an adjuvant to drug therapy in those cKCS dogs unresponsive to pharmacological treatment, in order to achieve analogue results of the responsive subjects (Moore et al., [94], Hendrix et al., [93]). Therefore, our investigation highlights the relevance of the possible administration of antioxidant/anti-inflammatory nutraceutical diet to cKCS dogs as useful adjuvant of immunosuppressive therapy.

The combination of a pharmacological treatment with a specific diet (Ocu-GLO Rx™) was also recently assessed by Williams et al. who successfully delayed the cataract formation in dogs with diabetes mellitus [104]. Specifically, the diet consisted in a mixture of a aldose reductase inhibitor, a glutathione regenerator alpha lipoic acid, grape seed extract, carotenoids, omega-3-fatty acids, and coenzyme Q10 which was provided to diabetic dogs as far as these developed lens opacification. Mean time without change in lens opacification was 278 ± 184 days with Ocu-GLO Rx™ and 77 ± 40 days in the placebo group.

In our treatement approach, the combination of several nutraceuticals, such as fish hydrolised proteins, rice carbohydrates, *Cucumis melo*, *Ascophyllum nodosum*, Astaxanthin, *Aloe vera*, *Carica papaya*, *Punica granatum*, *Camellia sinensis*, *Polygonum L.*, *Curcuma longa*, *Piper nigrum*, zinc and a omega3/6 polyunsaturated fatty acids (1:0.8 ratio), appears to exert beneficial immune-modulating effects on the clinical status of cKCS dogs. These data seams to confirm the action of nutraceutical diet on immune system modulation reducing Th1 and inproving TReg [35].

These plants and substances, widely used in traditional medicine, have been already shown to exert some intriguing antioxidant and anti-inflammatory activities in ocular tissues. In this regard, it is worth noting that *Camellia sinensis* extract was effective in conjunctival inflammation treatment [105] and *Curcuma longa* in several ocular diseases (chronic anterior uveitis, diabetic retinopathy, glaucoma, age-related macular degeneration and dry eye syndrome) [106, 107]. In addition, zinc was observed to reduce the progression of the age-related macular degeneration by the inhibition of the complement activation on retinal pigment epithelium cells [108] and omega 3 -6 fatty acids were closely correlated to development of vision and protection of eyes [109, 110].

The antioxidant/anti-inflammatory effects likely possessed by the mixture based on all these nutraceuticals in the diet supplementation seems to specifically reduce the immune-mediated ocular symptoms, particularly in those cKCS dogs that were poor responsive or unresponsive to classical immune-suppressive drugs.

In this regard, the pharmacological treatment alone was able to increase lachrymal production, while the increment was strongly higher and persistent when drugs were combined with the ND. Likewise, conjunctival inflammation was significantly reduced more in dogs belonging to ND group (receiving drugs in combination with nutraceutical supplemented diet) than in the SD group (receiving only the medical treatments). In addition, it is of relevance that corneal pigment density and mucus discharge were improved only in dogs belonging to the ND group. Finally, the occurrence of symptom relapsing, upon the suspension of nutraceutical diet, and of clinical amelioration, after its reintroduction, fosters the hypothesis of a possible therapeutical benefit of this nutraceutical diet in animal as well as in human ocular diseases [111, 112]

Taken in all, our results suggest that association of classical drug therapy with a nutraceutical diet with potential antioxidant/antiinflammatory and immune-modulating activities induce a significant amelioration of clinical signs and symptoms in keratoconjunctivitis sicca. Moreover, all symptoms appeared dependent on immune-mediated mechanisms. In this regard, the lachrymation impairment can be altered by an inflammatory condition of lachrymal gland and related ducts.

Therefore, it is reasonable to hypothesize that metabolic changes could affect immune response orchestration in a model of immune-mediated ocular disease. as represented by keratoconjunctivitis sicca, in dogs and, in a translational perspective, by Sjögren's syndrome in humans.

Study limitations

This research has some study limitations. For instance. neither the inflammatory cytokines present in the serum of dogs affected by KCS nor the percentage of regulatory T cells in the blood were evaluated. Ongoing experiments are characterizing the inflammatory cytokine release as well as the presence of Treg cells in peripheral blood. Moreover, preliminary results have evidenced that it is really hard to find in blood those alterations likely present in a well-defined peripheral tissue and body district, as represented by the eye.

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Authors' contributions
The contributions of the authors are as follows: SD, DG, MCM, SaC, SC, GG, GT participated in study design. SD, DG, MCM conducted the research. SD, DG, MCM, SaC, ADC, GT performed data interpretation. SD, DG, MGM. Sac, ADC and GT discussed the results and wrote the paper. SC and GG formulated the original idea. All authors read and approved the final manuscript.

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Competing interests
None of Authors has financial or personal relationships with other people or organizations and data interpretation was totally free from specific interests and the study has not been conditioned by any bias that could affect the results. This research was performed in collaboration with some scientists from the Division of Research and Development, Sanypet SpA, Padova, Italy (as indicated in the Author's affiliation) according to scientific and ethical principles of the scientific community. No financial funding was obtained from Sanypet Industry for this research study.

Consent for publication
Not applicable.

Ethics approval and and consent to participate
Operative procedures and animal care were performed in compliance with the national and international regulations (Italian regulation D.L. vo 116/1992 and European Union regulation 86/609/EC). The recommendations of CONSORT 2010 Statement in randomized controlled trials were also consulted and considered [113]. Written informed consent was obtained from the owners. A copy of the written consent is available for review by the Editor in Chief of this journal. Moreover, given that all procedures were part of routine care rather than an experimental intervention, an independent approval from an ethical committee was not necessary.

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Opportunities for topical antimicrobial therapy: permeation of canine skin by fusidic acid

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Abstract

Background: Staphylococcal infection of the canine epidermis and hair follicle is amongst the commonest reasons for antimicrobial prescribing in small animal veterinary practice. Topical therapy with fusidic acid (FA) is an attractive alternative to systemic therapy based on low minimum inhibitory concentrations (MICs, commonly <0.03 mg/l) documented in canine pathogenic staphylococci, including strains of MRSA and MRSP (methicillin-resistant *Staphylococcus aureus* and *S. pseudintermedius*). However, permeation of canine skin by FA has not been evaluated in detail. This study aimed to define the degree and extent of FA permeation in canine skin in vitro from two sites with different hair follicle density following application of a licensed ophthalmic formulation that shares the same vehicle as an FA-betamethasone combination product approved for dermal application in dogs. Topical FA application was modelled using skin held in Franz-type diffusion cells. Concentrations of FA in surface swabs, receptor fluid, and transverse skin sections of defined anatomical depth were determined using high-performance liquid chromatography and ultraviolet (HPLC-UV) analysis.

Results: The majority of FA was recovered by surface swabs after 24 h, as expected (mean ± SEM: 76.0 ± 17.0%). FA was detected within 424/470 (90%) groups of serial sections of transversely cryotomed skin containing follicular infundibula, but never in 48/48 (100%) groups of sections containing only deeper follicular structures, nor in receptor fluid, suggesting that FA does not permeate beyond the infundibulum. The FA concentration (mean ± SEM) in the most superficial 240 μm of skin was 2000 ± 815 μg/g.

Conclusions: Topically applied FA can greatly exceed MICs for canine pathogenic staphylococci at the most common sites of infection. Topical FA therapy should now be evaluated using available formulations in vivo as an alternative to systemic therapy for canine superficial bacterial folliculitis.

Keywords: Canine, Skin, Topical therapy, Pyoderma, Fusidic acid

Background

Antibiotic resistance is a major threat to global health and modern medicine [1]. Canine pyoderma caused by *Staphylococcus pseudintermedius* is amongst the commonest reasons for prescribing antimicrobial drugs in small animal veterinary practice [2]. Traditionally in canine practice, surface infections (confined to the interfollicular epidermis) are treated topically, whereas superficial infections such as bacterial folliculitis (that extend to the follicular infundibulum without extension into the surrounding dermis) are treated with oral antibiotics. The recent emergence of methicillin-resistant *S. pseudintermedius* (MRSP) [3] that are routinely resistant to licensed oral antibiotics has renewed interest in the direct application of topical antibiotics and antiseptics for superficial pyoderma [4, 5].

Fusidic acid (FA) is an antibiotic which has a steroid-like structure, with proven activity in vitro against coagulase-positive staphylococci including MRSP [6, 7]. The physico-chemical properties of this large, lipophilic molecule (molecular weight of 517 kDa, octanol/water partition co-efficient >4, 6 free hydrogen bonding groups) predict limited diffusivity through stratum corneum and restricted partitioning to the more hydrophilic

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living epidermis, after topical application [8, 9]. These features correlate with clinical efficacy of licensed FA-containing topical veterinary products in surface infections such as canine acute moist [pyotraumatic] dermatitis [10]. Utility in canine superficial pyoderma, however, is dependent upon adequate permeation into hair follicles, but this has received little attention. Studies of clinical efficacy of topical FA in canine superficial pyoderma / bacterial folliculitis are lacking [4, 11].

Stuttgen and Bauer established that in sparsely-haired human skin, FA is limited to the stratum corneum and epidermis after topical gel application, and does not penetrate into the deep dermis or subcutaneous fat [12]. By contrast, Degim et al. reported that 1.3% of FA in a betamethasone-containing gel formulation penetrated full-thickness haired canine skin in diffusion cell studies [13]. Skin integrity was not assessed prior to gel application, and FA was quantified in only receptor fluid and not on or within skin itself [13].

In view of these prior conflicting and incomplete data, we developed an in vitro model of topical FA application using canine skin held in Franz-type diffusion cells and high-performance liquid chromatography and ultraviolet (HPLC-UV) analysis of FA concentrations to define the degree and extent of drug permeation in skin from sites with varying hair follicle density. We describe for the first time how the depth of drug permeation into dermal layers can be defined by concurrent observation, in representative paired transverse histological sections [14, 15], of the variations in hair follicle anatomy that mark the infundibulum, isthmus and inferior portions of hair follicles. In addition, conventional analyses of drug recovery in receptor fluid and swabs from surface of dosed skin complemented evaluation of dermal drug concentrations. These data were used to inform likely clinical utility in canine superficial and deep pyoderma.

Methods

HPLC-UV detection of fusidic acid.

Validation

Fusidic acid sodium salt (≥98%, Sigma-Aldrich, Irvine, UK) was diluted in absolute ethanol to produce standard (0.5–49 μg/ml) and quality control (0.5, 1.0, 6.5 and 40 μg/ml) solutions (see Additional file 1 for chemicals used). High Performance Liquid Chromatography – ultraviolet analysis (HPLC-UV) was performed using an Ultimate 3000 (Thermo Scientific, Paisley, UK) system comprising quaternary pump, autosampler, column oven and diode array detector. The column was from Kinetex (C18 2.1 mm × 50 mm, 1.7 μm particle size; Phenomenex, Macclesfield, UK) held at 35 °C. Mobile phase A comprised methanol; mobile phase B 0.1 M acetic acid. Mobile phase A/B was ramped from 30/70 to 78/22 *v*/v ratio over 4 min and then held for 5 min. Mobile phase A/B then returned to 30/70 over 30 s and re-equilibrated for 7 min. The flow rate was maintained at 0.35 ml/min. The retention time of FA was 9.1 min, with UV detection at 240 nm.

Samples were analysed using a validated method developed at the University of Hertfordshire in accordance with OECD guidelines for studies of skin absorption in vitro [16]. Injection volume was 2 μl. The linear dynamic range for FA, based on peak areas with $1/x^2$ weighted regression was 1.24–249 ng on column ($R^2 = 0.9998$), with limit of detection (LOD) of 0.50 ng on column (signal/noise = 3). System precision, determined using replicate injections ($n = 10$) at 1.24 and 187 ng on column, was 6.6 and 0.76%, respectively. Receptor fluid, cotton wool swab and canine cryosection extracts (from 8 different animals) demonstrated no matrix interference at the retention time of FA. The calibration range was 0.5–49 μg/ml. Satisfactory intra and inter-run accuracy (87–107%) and precision (±15%) was obtained at low (1.0 μg/ml), mid (6.5 μg/ml) and high (40 μg/ml) concentrations of the calibration curve and at the limit of quantification (LOQ, 0.5 μg/ml). Extraction efficacies of FA from spiked canine skin cryosections (at low, mid, and high QC levels, 8 replicates of each) using ethanol confirmed recoveries to be 98.7–101.3%. Stabilities of spiking solutions and spiked matrices were shown to be at least 2 weeks when refrigerated. All experimental samples were analysed within 14 days of refrigerated storage.

Analysis of samples

Standard solutions covering the calibration range were run at the start of each batch with low, mid and high QCs bracketing no more than 15 test samples. Batch sample data were accepted when the accuracy and precision of these met validation criteria. In order to obtain concentrations of FA found in skin samples, the amount of FA found in the skin was adjusted for the sample weight from which the sample was obtained.

Canine skin collection

Full thickness canine skin was obtained from healthy Beagle dogs (three male, three female, aged 6–12 months, 8–14 kg) immediately after euthanasia for reasons unrelated to this study (approved by the Royal Veterinary College's Clinical Research Ethical Review Board 2016 1651–2-R). Hair was clipped (Moser Arco 1854, Wahl, Sterling, IL, USA) to within 3 mm of skin surface taking care not to damage skin integrity, and skin was then excised from the dorsum and groin of each dog, immediately wrapped in tin foil and chilled by frozen ice blocks prior to storage at -20 °C within 6 h of collection. Harvested skin was used within 5 months [17].

Skin measurements

Thickness of whole skin specimens

The thickness of the centre of each 3 × 3 cm portion of skin was measured using callipers immediately before assembly into the diffusion cells, as described below.

Thickness of stratum corneum

Vertical cryosections through full thickness skin were prepared from undosed skin from each treatment group (undamaged, shampoo-treated and tape-stripped skin) for both dorsum and groin for all six animals (total n = 36). Eight cryosections were taken from each piece of skin, sectioning both from panniculus to epidermis (n = 4) and from epidermis to panniculus (n = 4), and stained with haematoxylin and eosin. Thickness of stratum corneum was measured at three points per section where stratum corneum was at its most compact / intact and not obviously folded [18] using a light microscope (×40 magnification) and Image-Pro Plus v5.0.1.11 software (Media Cybernetics, Duxford, UK).

Hair follicle density

Hair follicle density (compound follicles per mm^2) and infundibular area (as a percentage of skin area) at the level of the common infundibulum were compared in replicate control untreated dorsal (n = 6) and groin (n = 6) skin samples by microscopy of transverse haematoxylin and eosin-stained paraffin sections of skin from one male and one female Beagle dog. The hair follicle count and area were measured at three randomly selected areas per section using microscope settings and software as for stratum corneum measurements described above.

Electrical resistance

Electrical resistance between saline treated epidermal skin surface and receptor fluid was used to assess skin barrier integrity in each assembled diffusion cell using an ohmmeter (Iso-Tech LCR-821 Meter, Iso-Tech, Southport, UK) [19].

Diffusion cell experiment

Four dermal absorption experiments with full thickness canine skin were conducted using a 10 mg/g FA suspension (Isathal®, Dechra Veterinary Products (DVP), Shropshire, UK) which contains the same vehicle as a licensed topical skin product for dogs (Isaderm®, DVP). Dorsal and groin skin sourced from six animals (three dogs per experiment) was defrosted and defatted by blunt dissection before division into three evenly sized pieces (70 cm^2), one for each treatment group and assembled into diffusion cells containing receptor fluid within 3 h. For each experiment, 21 static Franz diffusion cells (Permgear Inc. Hellertown, PA USA) were assembled with portions of either dorsal or groin skin (3 cm × 3 cm) which had either been left untreated (n = 6), repeatedly tape-stripped (n = 6) to mimic damage to the stratum corneum, or shampooed with a 2% chlorhexidine and 2% miconazole shampoo (Malaseb®, DVP) to mimic clinical use (n = 6). One diffusion cell containing untreated skin from each animal was assembled but left undosed (negative controls). Skin allocated to be tape-stripped was quickly [20] and repeatedly (n = 30) [20] stripped with D-Squame discs (22 mm; Cuderm, Dallas, TX, USA) prior to assembly into the cells. A uniform pressure was applied to each disc for 2 s using a 225 g/cm^2 applicator before disc removal with forceps. The epidermal surface of relevant portions of excised skin (approximately 70 cm^2) were moistened and shampooed (0.02 ml/cm^2) for 2 min by hand, then left for 10 min, as per label instructions for clinical use, prior to rinsing with water (2 × 5 ml). Treated skin was blotted dry with paper towels and cut into pieces for assembly into the cells.

Each piece of skin was placed between a glass receptor chamber, containing measured volumes (approximately 14 ml) of ethanol / pH 5.0 phosphate buffered saline, 25/75 v/v [13], and a magnetic stirrer, and glass donor chamber and secured by pinch clamp, exposing 1.77 cm^2. Diffusion cells were randomly assigned positions in stirrer blocks and plumbed into a heated water circulator system in order to maintain a constant skin surface temperature of 32.0 ± 1.0 °C confirmed by infrared camera (P620, FLIR, West Malling UK). Skin barrier integrity was established in each cell by measurement of electrical resistance as described above.

After equilibration overnight, 18 of the 21 assembled cells were dosed with 100 µl of the FA suspension using a calibrated positive displacement pipette. Saline (0.9%, 100 µl) was added by pipette to all cells in order to liquefy the gel [13]. A glass rod was used to gently spread the gel across the entire exposed surface of skin. The total amount of FA applied to each cell was determined by weighing the filled and emptied pipette tip and the glass rod before and after use. The donor chambers were then promptly occluded using plastic paraffin film.

Receptor fluid samples (250 µl) were collected from each cell before (pre-dose) and at 12 and 24 h after dosing, with replacement of equal volumes of fresh receptor fluid at each time point. Additional sampling intervals were deemed unnecessary in anticipation of negligible penetration into the receptor fluid. After 24 h, the donor chamber was removed and the surface skin and inside of the donor chamber were both swabbed with cotton wool. Swabs were transferred to glass vials and soaked in ethanol (10 ml) for a minimum of 24 h at 4 °C to extract the FA. Aliquots of the swab extracts and receptor fluid samples were transferred to autosampler vials (2 ml) and crimped capped. Skin specimens were gently removed from the receptor chamber using forceps, taking care not to touch the exposed area of skin, and were stored

in foil at −70 °C prior to cryosectioning (to minimise drug lability in the skin).

FA concentrations in ethanolic swab extracts and receptor fluid samples were determined by direct injection of aliquots, transferred to autosampler vials, using the HPLC-UV method described above.

Skin cryosectioning

OCT-embedded frozen specimens of skin were cryosectioned transversely starting from the deep dermis proceeding towards epidermis in groups of seven sections, to avoid cross contamination of sections with the blade. Each group comprised a) five sequential 20 µm sections that were placed in individual glass vials and extracted in ethanol (5 ml) for 24 h, for subsequent FA HPLC-analysis, and b) a further two 10 µm sections which were mounted on Polysine™ slides (ThermoFisher Scientific, Paisley, UK) prior to staining with haematoxylin and eosin. The stained slides were examined by light microscopical observation of hair follicle anatomy and presence of other skin structures by a blinded assessor (RB) to determine the anatomical depth within the skin (Figure 1). As the highest proportion of hair follicle infundibula were present in the uppermost two vials (equivalent to a depth of approximately 240 µm) these were selected for determination of the maximum FA concentration achievable in the superficial skin layers in these experiments.

Statistical analyses

Statistical analyses utilised IBM SPSS Statistics Package 21 (IBM, Portsmouth, UK) with P values of ≤0.05 considered significant. Normality was assessed through Shapiro-Wilk test prior to use of either the Kruskal-Wallis test with Dunn's *post-hoc* test, or one-way ANOVA with Bonferroni *post-hoc* correction as appropriate. Chi-squared tests evaluate contingency table data.

Results

FA recovery

The amount of FA applied to canine skin in each diffusion cell ranged from 762 to 1087 µg (mean ± SEM 946 ± 9 µg). All QCs and standards running alongside samples met validation criteria. FA was never detected in any sample from un-dosed control cells, nor detected within quantifiable limits in any receptor fluid sample 24 h after application (Table 1). HPLC-UV analyses indicated that total FA recovery was 90.2 ± 9.0% (range 65–107%) after 24 h; no significant difference was found between skin sites or treatment groups (Table 1). From skin surface swabs, overall recovery of FA was 76.0 ± 17.7% independent of skin site or treatment group. A significantly ($P = 0.002$)

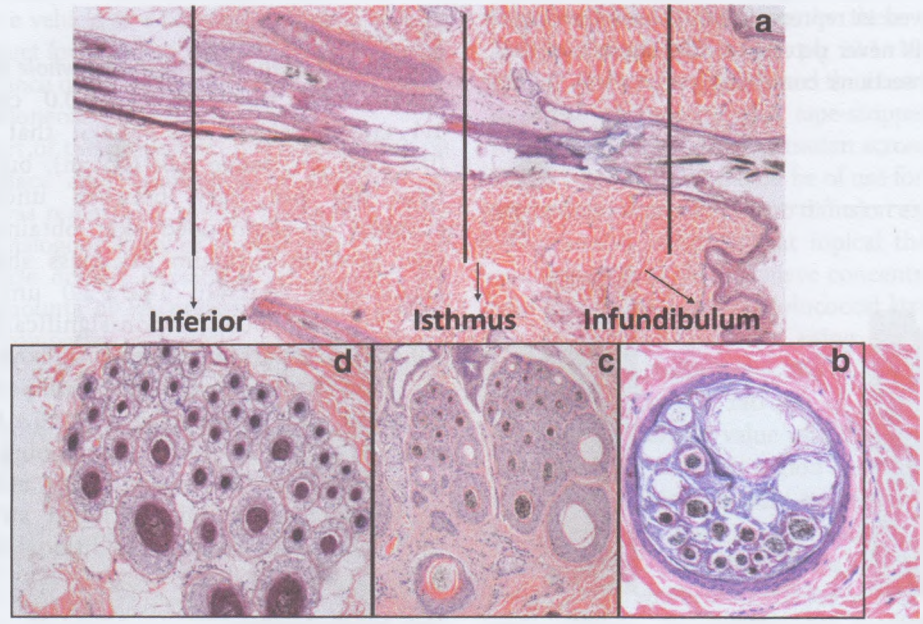

Fig. 1 Composite image of the histology of the canine compound hair follicle. **a** Traditional vertical section through the long axis of a compound follicle from epidermis (right) to panniculus adiposus (left). Lines indicate planes of section for corresponding transverse images that define depth of section. **b** Transverse section at common infundibulum: follicle is lined by stratified squamous keratinising epithelium that recapitulates that of the interfollicular epidermis and contains multiple naked hair fibres. **c** Transverse section at isthmus: compound follicle comprises a cranial primary hair and a group of (commonly 14–18) secondary hair follicles; each hair shaft is surrounded by root sheaths whose anatomy varies with stage of hair growth. **d** Transverse section at inferior portion of follicles: presence indicates anagen phase represented by hair fibre surrounded by inner root sheath and glycogen-rich outer root sheath

Table 1 Mean (±SEM) individual and combined percentage recoveries of FA

Measurement (%)		Site	Treatment Group		
			Undamaged	Shampooed	Tape-stripped
Site of FA recovery	Total drug	Dorsum	87.2 ± 6.8	93.0 ± 6.7	92.9 ± 3.6
		Groin	89.2 ± 5.4	89.2 ± 7.8	89.9 ± 6.0
	Surface swab	Dorsum	60.4 ± 15.8	80.5 ± 10.0	79.2 ± 6.0
		Groin	77.2 ± 5.9	78.5 ± 12.0	80.4 ± 6.4
	Skin cryosections	Dorsum	26.9 ± 10.2	12.6 ± 4.0	13.7 ± 2.7
		Groin	12.0 ± 3.3	10.7 ± 4.5	9.4 ± 3.6
	Receptor fluid	Dorsum	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		Groin	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

FA: fusidic acid. Mean (±SEM) individual and combined percentage recoveries of FA from in vitro diffusion cells containing dorsal or groin skin from healthy Beagle dogs ($n = 6$) after topical application of a 10 mg/g FA suspension (Isathal®) for 24 h. Skin samples were undamaged, shampooed or tape-stripped ($n = 6$ per group) prior to dosing. After exposure, the skin was swabbed prior to transverse cryosectioning and swabs, skin samples and receptor fluid analysed for FA content

higher percentage of the applied dose was found within cryosections from the dorsum (17.7 ± 2.4%) in comparison to the groin (10.7 ± 1.0%; Figure 2).

No significant inter-dog variability in FA distribution was seen between skin from the six donor dogs within skin group (undamaged, shampooed or tape-stripped) or site (Additional file 2).

FA was detected in 80% of vials (376 of 470 vials, equivalent to 72.6% of all 518 vials; Table 2) containing cryosections where follicular infundibula or more superficial structures (surface hairs, living epidermis, keratin and free hairs) were observed in representative (paired) histological specimens. FA was never detected in the absence of these structures, i.e. in sections containing only isthmi, inferior portions of the hair follicle or subcutaneous fat ($n = 48$ vials; Table 2; $P < 0.0005$, Chi-squared test).

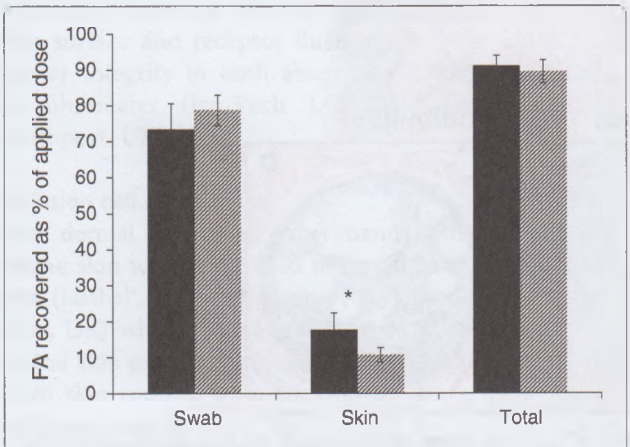

Fig. 2 Individual / combined percentage recoveries of FA (mean ± SEM) in skin and swab following 24 h topical application of a 10 mg/g carbomer gel formulation of FA to dorsal and groin skin from healthy Beagle dogs

A mean (± SEM) FA concentration of 395.4 ± 30.9 µg/g was found in each treated portion of skin 24 h after topical application (Table 3). No significant differences ($P > 0.05$) were seen between the concentrations achieved in any site or treatment group. The concentration of FA (mean ± SEM) in the uppermost (from epidermal aspect) two vials of cryosections of each skin specimen (approximately 240 µm) was 2000 ± 815 µg/g.

Skin measurements

Thickness of stratum corneum and whole skin specimens

Dorsal skin thickness (1.1 ± 0.0 cm; range 0.9–1.4 cm) exceeded ($P < 0.0005$) that of groin skin (0.8 ± 0.0 cm; range 0.5–1.1 cm), but overall thickness did not vary between undamaged, tape-stripped or shampooed skin obtained from either site. Stratum corneum thickness showed a similar relationship (dorsum 14.0 ± 2.0 µm; groin 12.0 ± 1.6 µm; $P < 0.0005$). A non-significant reduction ($P = 0.105$) in stratum corneum thickness was seen in the tape-stripped groin skin compared to the undamaged skin (undamaged 12.6 ± 1.9 µm, tape-stripped 11.7 ± 1.1 µm); shampooing had no measureable effect. Sectioning from either dermal or epidermal aspects did not affect stratum corneum thickness measurements (Additional file 3).

Hair follicle density

The hair follicle density of dorsal skin (mean ± SEM, 5.8 ± 0.2 compound follicles/mm^2) exceeded ($P = 0.004$) that of groin skin (2.2 ± 0.6 compound follicles/mm^2), with a greater percentage of the total sectioned area of skin containing follicular infundibula (dorsum 19.8 ± 1.8%, groin 6.7 ± 1.6%; $P = 0.004$).

Table 2 Comparison between presence of infundibular and more superficial structures and presence of FA

	Vials with FA detected n (%)	Vials without FA n (%)
Infundibulae or surface structures on sections	376 (72.6)	94 (18.1)
No infundibulae or surface structures on sections	0 (0.0)	48 (9.3)

FA: fusidic acid; $P < 0.0005$, Chi-squared test. Topical application of a 10 mg/g FA suspension (Isathal®) for 24 h to dorsal or groin skin from healthy Beagle dogs ($n = 6$) held in diffusion cells; a comparison between the presence of infundibular and more superficial structures (surface hairs, interfollicular epidermis, keratin and free hairs) versus deeper structures (isthmi,inferior portions, subcutaneous fat) on analysis of histological specimens and the presence of FA in the corresponding vials of cryosections (vials, $n = 518$;) when assayed by HPLC-UV

Electrical resistance

Barrier integrity testing prior to analysis demonstrated that electrical resistance was comparable ($P = 0.452$) between the dorsum and groin skin (dorsum mean ± SEM = 3.71 ± 0.19 kΩ, groin = 3.46 ± 0.16 kΩ).

Discussion

The combination of transverse histological sectioning and HPLC-UV assessment of FA concentrations in serial sections was pivotal in ascertaining in detail the depth of FA permeation through canine skin. Coupled with the more conventional processes of drug recovery in post-treatment skin, surface swab and receptor fluid samples, these data confirmed FA permeation to the level of the follicular infundibulum and thus, the presence of drug at the level of infection in canine superficial bacterial folliculitis following topical application. Whilst formulation with different vehicles commonly influences skin permeation, we used a commercially available ophthalmological product that contains the same vehicle as a licensed steroid-containing topical skin product for dogs (Isaderm®, DVP) to maximise the clinical relevance of the results of our in vitro study to veterinary practitioners.

This first report of the proportion of FA remaining on canine skin surface after application in vitro (76.0 ± 17.7% at 24 h) was remarkably similar to the 80% figure reported in an analogous study of human skin [12]. The higher hair follicle density dorsally likely accounts for the increased amounts of FA present within skin from this site when compared with groin, particularly since the presence of drug in skin sections was significantly associated with histological observation of infundibulae and other superficial structures.

Table 3 Mean (± SEM) concentration of FA measured in transverse cryosections

FA concentration (mean μg/g)		Treatment Group		
		Undamaged ($n = 6$)	Shampooed ($n = 6$)	Tape-stripped ($n = 6$)
Site	Dorsum	617.5 ± 135.9	345.0 ± 46.0	346.4 ± 35.4
	Groin	392.3 ± 51.8	298.8 ± 47.9	372.4 ± 69.0

Mean (± SEM) concentration of FA measured in transverse cryosections obtained from full thickness dorsum or groin skin from healthy Beagle dogs ($n = 6$) after topical application of a 10 mg/g FA suspension (Isathal®) for 24 h in four diffusion cell experiments. Skin samples were undamaged, shampooed or tape-stripped ($n = 6$ per group) prior to dosing. After exposure, the skin was swabbed prior to transverse cryosectioning. No significant differences were seen between the concentrations achieved in any site or treatment group (ANOVA, $P > 0.05$)

The failure to detect FA in receptor fluid in this canine study was in accordance with a previous in vitro penetration study of human skin [12], and not un-expected from the physicochemical properties of the molecule [8, 9]. The limit of detection of this HPLC method was well below the predicted concentration of FA in receptor fluid had we reproduced the 1.3% bioavailability described by Degim et al [13]. The full thickness penetration of FA reported in that study might reflect technical or procedural differences such as apparent absence of barrier integrity testing prior to dosing; [13] ensuring that the epidermal barrier layer maintains its integrity is an essential factor to the successful performance of diffusion cell experiments [16]. In this study, electrical resistance was used for barrier integrity testing, but this does not appear to have been described previously for dogs. Values obtained here fell between those reported for rat (3 kΩ) and pig (4 kΩ) skin [19], in parallel with relative stratum corneum thickness in these species (rat 6.0–13.3 μm < dog 9.4–15.1 μm < pig 13.1–18.1 μm) [21]. Comparable electrical resistance and thus barrier integrity in undamaged, shampooed and tape-stripped skin correlated with the equivalent FA penetration across the three groups. Values reported here should be of use for future skin integrity testing for canine in vitro diffusion experiments.

Our model indicates that topical therapy with FA in canine skin is likely to achieve concentrations that markedly exceed MICs of staphylococcal strains deemed both 'susceptible' and 'resistant' using existing interpretative criteria. By extrapolating the mean FA concentration achieved in the top 240 μm of skin (2000 ± 815 μg/g) using a skin density value of 1.09 [22], the overall concentration of FA in this region can be estimated as 2180 ± 634 mg/l. This markedly exceeds previously reported MIC_{90} of both methicillin-resistant and susceptible *S. aureus* and *S. pseudintermedius* [6, 23], and EUCAST systemic therapy breakpoint for 'resistance' (1 mg/l) [24] and compares favourably with MIC_{100} values for FA-resistant MRSA (1024 mg/l) [7]. Development of interpretive criteria for topical rather than just systemic use of antimicrobial therapy is urgently required.

The stratum corneum thickness of undamaged canine skin in this study was closely comparable to those of previous reports [21, 25]. Tape-strip removal of stratum

corneum cells and lipid is commonly used to degrade the barrier and enhance drug permeability, although post-stripping measurements of thickness in cryosections (which best preserve stratum corneum architecture in haired skin) [21, 26] are very rarely reported [27–29]. We speculate that the failure of tape stripping to significantly reduce canine interfollicular stratum corneum thickness reflects the combined effects of a dense mat of short stubbly hairs reducing D-squame tape access to the interfollicular epidermis (close clipping was avoided to prevent stratum corneum disruption) [29], uneven skin surface [30], thicker corneum at follicular ostia [31], and or preferential removal of loose corneocytes that may be lost or otherwise not included in measurements of residual compact layers. Further studies that optimise parameters, such as applicator pressures, numbers of repeat strips and clipping methods [20] for the thin but compact corneal layers of canine haired skin, are indicated.

Conclusions

These data suggest that topical FA should be useful in the treatment of canine surface and superficial pyoderma (intact follicles) caused by bacteria susceptible to fusidic acid, in countries where it is available, but not deep pyoderma (where infection extends to surrounding dermis). Clinical studies are now required to confirm this. Similar studies should now be performed for other topically applied antibiotics to inform evidence-based antibiotic treatment guidelines. Although prevalence of antimicrobial resistance should be monitored prospectively, FA provides an opportunity for topical antibiotic therapy in the treatment of staphylococcal folliculitis in dogs and an option to reduce selection pressure for antimicrobial resistance on these zoonotic canine pathogens from conventional systemic antibiotic use.

Additional files

Additional file 1: Chemicals used in diffusion cell analysis of fusidic acid permeation into canine skin.

Additional file 2: Mean ± SEM percentage of applied dose of fusidic acid recovered. Description of data: Mean ± SEM percentage of applied dose of fusidic acid recovered from swab, within skin or in total for Beagle dogs ($n = 6$) after topical application to undamaged, shampooed or tape stripped dorsum or groin skin.

Additional file 3: Mean (± SEM) thickness of stratum corneum measured on vertical cryostat sections. Description of data: Mean (± SEM) thickness of stratum corneum measured on vertical cryostat sections, cut from panniculus up to epidermis or epidermis down to panniculus. Sections taken of full thickness dorsum or groin skin from healthy Beagle dogs ($n = 6$) treated in three ways: undamaged, shampooed or tape-stripped ($n = 6$ per group).

Abbreviations

DVP: Dechra Veterinary Products, Shrewsbury, U.K.; FA: Fusidic acid; HPLC-UV: High pressure liquid chromatography – ultraviolet; LOD: Limit of detection; LOQ: Limit of quantification; MIC: Minimum inhibitory concentration; MRSP: Methicillin-resistant *Staphylococcus pseudintermedius*; OCT: Optimum cutting temperature; QC: Quality control; SEM: Standard error of the mean

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Authors' contributions

All authors contributed to concept and design of this study; SMF and JL conducted the experiments and analysed data; RB and SMF performed the histological examination of skin samples; all authors contributed to writing the manuscript and approved the final manuscript.

Consent for publication

Not applicable.

Competing interests

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Pilot evaluation of a novel unilateral onychectomy model and efficacy of an extended release buprenorphine product

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Abstract

Background: Non-steroidal anti-inflammatory drugs (NSAIDs), transdermal fentanyl patches, and transmucosal buprenorphine are probably the most commonly used options for providing post-operative analgesia in the early at-home period. However, these require daily administration or are associated with abuse concerns. One of the significant unmet needs in veterinary surgery and pain management is for longer acting opioids for cats to effectively bridge the gap between the in-hospital and at-home recovery periods.

A proof of concept study of an extended release formulation of buprenorphine HCL (ER-Bup) was conducted using objective kinetic measures and a unilateral onychectomy model. Using a blinded, randomized, two period crossover design, four cats were allocated to control (saline) or ER-Bup (0.6 mg/kg, subcutaneously [SC]) treatment groups. All animals underwent a unilateral forelimb onychectomy per period with a washout/recovery period in between. Observational pain scores and kinetic data (using a pressure sensitive walkway [PSW]) were collected prior to (baseline) and at intervals for 72 h following surgery. Symmetry indices were derived for kinetic variables (peak vertical force [PVF]; vertical impulse [VI]) of each forelimb for landing following a jump and for walking. A rescue analgesic protocol was in place. Effect of surgery and treatment were evaluated using a mixed model statistical approach.

Results: No cats required rescue analgesics based on subjective pain score. ER-Bup had a positive influence on subjective pain scores during the 72 h postsurgery ($p = 0.0473$). PVF and VI of the operated limb were significantly decreased for both landing ($p < 0.0001$ and $p < 0.0001$) and walking ($p < 0.0001$ and $p < 0.0001$ respectively) compared to control. ER-Bup resulted in significantly decreased asymmetry in limb use during landing (PVF, $p < 0.0001$; VI, $p < 0.0001$) and walking (PVF, $p = 0.0002$, VI, $p < 0.0001$). The novel use of data collected following a jump from an elevated platform appeared to provide all desired information and was easier to collect than walking data.

Conclusion: This study demonstrates that SC administration of ER-Bup may be an effective analgesic for a 72 h period postoperatively. Furthermore, landing onto a PSW from an elevated perch may be a useful and efficient way to assess analgesics in cats using a unilateral model of limb pain.

Keywords: Buprenorphine, Extended release, Pressure sensitive walkway, Landing, Cat, Kinetic

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Background

Inadequate control of pain in the perioperative period in cats has been linked to poor recoveries, postoperative complications and potentially chronic pain [1]. Non-steroidal anti-inflammatory drugs (NSAIDs), transdermal fentanyl patches, and transmucosal buprenorphine are probably the most commonly used options for providing post-operative analgesia in the early at-home period [2]. However, there are concerns about side effects of NSAIDs; there are potential abuse concerns with sending fentanyl patches into the home environment [3]; and transmucosal buprenorphine is not always easy for owners to accomplish [2]. One of the significant unmet needs in veterinary surgery and pain management is for longer acting opioids for cats to effectively bridge the gap between the in-hospital and at-home recovery periods.

Buprenorphine, a synthetic opioid drug that is classified as a partial μ opioid receptor agonist, is commonly used for pain management in cats via multiple administration routes [4, 5]. This drug reportedly has a strong affinity for, and dissociates slowly from, the mu opioid receptors [6]. Because of this pharmacodynamic profile, the immediate release formulation of buprenorphine is considered to be one of the longest-acting opioids available with a duration of action greater than 6 h [7, 8]. Additionally, the incidence of undesirable side effects, such as vomiting, nausea, respiratory depression and dysphoria, is reportedly lower compared to other opioids [2]. Due to these characteristics, it has become one of the most popular opioid analgesics for use in cats in many countries [9, 10]. A recent clinical study, however, did illustrate that cats undergoing ovariohysterectomy may require a second dose of buprenorphine 4 h after surgery [11]. This, and other data, suggests that a single-injection is not sufficient for effective postoperative pain relief [5, 12].

It is well established that it is difficult to recognize and measure pain in cats [1, 13]. Multiple methods attempting to accurately assess pain have been described [14, 15], and several research groups are in the process of developing and validating subjective pain scales for clinical use [14, 16–18]. One objective method to evaluate pain is gait analysis, which is only relevant if the origin of the pain affects gait. Pressure sensing platforms, or pressure sensitive walkways (PSW) have been investigated for the measurement of limb use in cats [19, 20] and used for the evaluation of limb use following onychectomy [21]. It is difficult to collect high quality limb use data when relying solely on cats freely walking across a PSW in a straight line (unpublished observations).

Several years ago kinetic evaluation of jumping cats using a PSW was proposed by our pain research group at NC State [20] and recently revisited by another team of researchers [19]. One potential use of this method is to measure the left-right difference in kinetic parameters when there is unilateral forelimb pain.

The availability of an extended release formulation of buprenorphine (ER-Bup) may provide effective analgesia in the immediate postoperative period in the hospital and at-home. Evaluation of an extended release formulation of buprenorphine in dogs undergoing arthrotomy provided pain relief over a 72 h period [22]. In cats, it has been reported that the administration of a single dose of ER-Bup had a similar efficacy (as evaluated by subjective pain assessment scales) and adverse effect profile as twice-daily oral transmucosal administration of buprenorphine [23]. However, there was no placebo control group in this study, and the investigators did not use a validated subjective assessment tool, and so the efficacy of the new formulation of buprenorphine is unknown.

The aim of the present study was to perform a pilot proof of principle pilot study of ER-Bup using objective kinetic measures collected from walking, and landing following a jump from an elevated perch (0.7 m), in a unilateral onychectomy model. It was hypothesized that unilateral onychectomy would result in asymmetry of forelimb kinetic values in cats, and that ER-Bup would decrease this asymmetry. Further, it was hypothesized that collecting data from cats landing from an elevated platform (jumping down) would result in measureable objective parameters similar to the classical collection of kinetic variables in freely ambulating cats.

Methods

Study design

The study was an experimental, masked, randomized, placebo controlled, two period cross-over design. The investigator, surgeon and any others involved in making assessments regarding product efficacy or safety were unaware of treatment assignment. The study monitor and dose administrator were un-masked but were not involved in clinical assessments. The cats were housed in a climate-controlled facility in compliance with the U.S Animal Welfare Act. All study procedures were approved by the Institutional Animal Care and Use Committee (protocol number PLRS 1006). The study was initiated at Professional Laboratory and Research Services, Inc.

Animals

Four purpose-bred mature domestic short-hair sexually intact two male and two female cats were used. They were considered healthy based on physical examination, complete blood count, serum biochemical analysis, and urinalysis. Body weights ranged from 2.2 to 3.2 kg. Age of all cats was approximately 10 months old. The cats were housed individually in a room with a controlled environment and were acclimated to their surroundings for at least 30 days.

Treatment and surgical procedures

Treatment groups consisted of a control group (0 mg/kg; saline) and ER-Bup group (0.6 mg/kg subcutaneously, SC). In previous work (unpublished kinetic studies) 0.6 mg/kg of ER-Bup was confirmed not to cause significant ataxia, weakness or other impairment that might be interfere with ability to assess gait or other treatment during the development of this product. The cats were randomly allocated to one of the two treatments for each limb. A two period crossover study was employed to evaluate the two groups as shown in Table 1. All animals underwent two onychectomy surgeries; the first foot during period 1 and the other foot during period 2 with a washout/recovery period between (Fig. 1). Cats were deemed ready for a second surgery when peak vertical force measurements (PVF), both at a walk and following landing from an elevated platform, were within the 95% confidence interval of the average of the two baseline PVF measurements. The same anesthetic regimen and surgical procedure was used in each period. As previously described [24], guillotine-type nail clippers were carefully positioned to completely remove the third phalanx. Treatments (saline or ER-Bup) were administered 20–60 min prior to anesthetic induction. Four weeks after the first surgery, during the washout/recovery period, kinetic variables were collected at pre-determined intervals and analyzed. Upon the conclusion of the study, each cat had had both forelimbs declawed with one foot being done with the test article being incorporated into the pre-operative anesthetic procedure and the other being done with a negative control.

Anesthesia protocol

Pre-anesthetic medication with acepromazine at 0.03 mg/kg intramuscularly 20–60 min prior to anesthetic induction (administered at the same time as the ER-Bup or saline). Intravenous propofol (2–6 mg/kg) was administered to effect to induce general anesthesia. Isoflurane carried in O_2 was administered via an endotracheal tube for the maintenance of anesthesia. During anesthesia, cats were monitored for heart rate, respiration rate, temperature, end tidal CO_2, oxygen saturation, and blood pressure was measured noninvasively. Vital signs and comfort were periodically assessed after anesthesia until the cats were fully recovered from anesthesia and their rectal temperature was normal.

Table 1 Animal allocation and randomization schedule

Cat	Treatment sequence	Period.1		Period.2	
		Test article	Foot	Test article	Foot
1	1	Saline	L	ER-Bup	R
2	1	Saline	R	ER-Bup	L
3	2	ER-Bup	R	Saline	L
4	2	ER-Bup	L	Saline	R

ER-Bup extended release formulation of buprenorphine HCL

Collection of kinetic variables using a Pressure Sensitive Walkway (PSW)

A PSW (7100 QL Virtual Sensor 4Mat System, Tekscan, Boston, MA), 2.4 m × 0.5 m with 4 sensels/cm^2, was used to collect kinetic data from walking and landing cats as described previously [20].

The data were recorded at 6, 24, 30, 48, 54, and 72 h after surgery. Baseline gait analysis was performed for each animal 24 and 48 h prior to each surgical procedure (Fig. 1). A 50 psi sensor range was selected following a review of previous work that indicated this was the most appropriate sensor range [20]. The output data recorded was analyzed using proprietary Tekscan software (Walkway v7.0 software, Tekscan, Boston, MA). Each day, the whole area of the pressure platform was loaded and unloaded by walking on it (conditioning), and it was then equilibrated and calibrated according to the manufacturer's instructions. For all data collection, the frame acquisition rate was set to the maximum frequency of 60 Hz. All cats underwent daily walking and jumping down training on the PSW for approximately 30 days prior to study initiation. Following the washout/recovery period training sessions were again performed for 12 days prior to the second surgery. For the collection of walking data, valid data movies were defined as the cat traversing the PSW in a straight line, within individualized velocity and acceleration parameters, with no visually detectable movement of the head from side to side, and no visually detectable slowing down or acceleration. Cats were encouraged to traverse the mat spontaneously using treats and toys. Cardboard barriers were sometimes used on either side of the PSW to prevent the cats from wandering off the mat. Ten sets of walking data were collected, with at a target velocity of 0.6 ± 0.2 m/s and within acceleration changes of ±0.1 m/s^2. Either using manual analysis or automated analysis available within the software, the velocity of each 'run' was calculated to ensure there were ten data sets collected within the velocity parameters set. As described later, post-collection analysis used the five data sets that were closest to the target velocity and acceleration parameters to minimize the impact of differences in velocity on kinetic parameters. Additionally, at each time point, ten sets of data were collected with the cats landing on the mat after jumping down from an elevated platform. The cats were encouraged to jump down onto the PSW, using treats and toys, from an elevated platform that was 0.7 m vertical distance from the ground (and PSW), landing on the PSW with their forelimbs first (landing data). Valid data movies were defined as those where cats jumped down

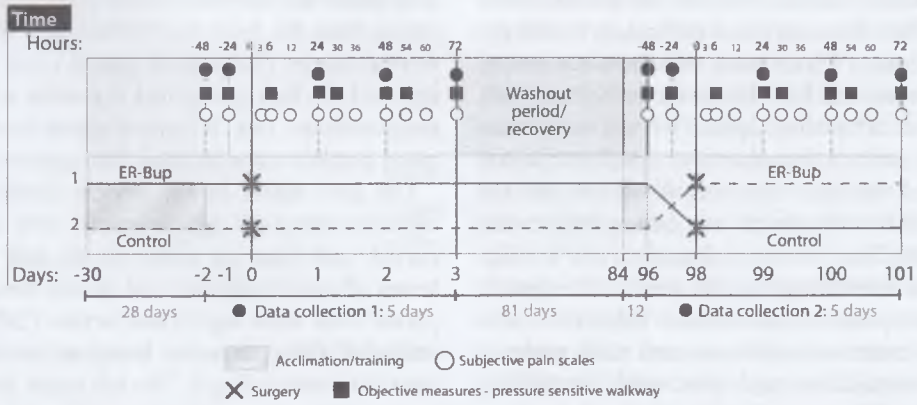

Fig. 1 Schematic outline of the study protocol. Using a blinded, randomized, two period crossover design, four cats were allocated to control (saline) or ER-Bup (0.6 mg/kg, subcutaneously) treatment groups, in random order. The cats underwent a unilateral forelimb onychectomy per period with a washout/recovery period between. Observational pain scores and kinetic data were collected prior to (baseline) and at intervals for 72 h following surgery (total of 5 days of data collection). All cats underwent daily walking and jumping training on the PSW for approximately 30 days prior to study initiation. Following the washout/recovery period training sessions were again performed for 12 days prior to surgery. The washout/recovery period was 81 days

in a forward direction, and landed with the forelimb(s) first. Using the software, kinetic data were collected from each forelimb footfall for each of five valid trials. PVF and vertical impulse (VI) were collected. All forces were normalized to and expressed as a percentage of the cat's body weight. Forelimb symmetry indices (SI) for PVF and VI, during both walking and landing were calculated by use of the following equation [25].

$$SI = \frac{(x_{op} - x_{no})}{(1/2)(x_{op} + x_{no})} \times 100$$

Where x_{op} is the mean of a given gait variable for operated limb and x_{no} is the mean of a given gait variable for non-operated limb. Time between first and second forelimb strike (Tf_1f_2) was calculated by subtracting time of contact of the non-operated limb from time of contact of the operated limb. The number of trials where there was a measurable difference in the time of first and time of second forelimb strike (NTf_1f_2) was counted. Time between first forelimb and first hindlimb strike (Tf_1h_1) was calculated by subtracting time of contact of first forelimb from time of contact of first hindlimb.

Rescue analgesia

Observational pain assessments were completed at 3, 6, 12, 24, 30, 36, 48, 54, 60, and 72 h after treatment (Fig. 1) using a subjective scoring system (Additional file 1). Baseline assessments were made for each animal 24 and 48 h before initial and second surgery. A priori, it was decided that any cat that with a pain score of four at any time would be administered rescue analgesia. Pain scores were assigned by a single trained observer. For cats requiring rescue analgesia, meloxicam was administered at a dose of 0.1 mg/kg SC.

Statistical analysis

Statistical analyses were performed using JMP and SAS (JMP Pro, version 11, SAS 9.4, SAS Institute, Cary NC). Wilcoxon signed-rank test and a paired t-test were used to compare the area under the curve minus baseline (AUC) of pain score versus time between groups. For pain score versus time curves a logistic regression with repeated measures analysis for time and maximum likelihood fitting method was used (PROC GLIMMIX in SAS). Pain score was treated as an ordinal variable in a logistic model with treatment group, period, time as main effects and with interaction terms of time*treatment and period*time (See Additional file 2). The GLIMMIX output also calculated the probability of a score of baseline versus time for both periods and for both treatment groups. A predicted time at which a probability of 0.5 was reached, is similar to an LD50, was obtained (Fig. 3).

Mixed model analysis was conducted to test the effect of time on the outcome measures in the control group in order to see if the cats undergone onychectomy had symmetrical gait. Cat was included as a random effect in the mixed model. Within each treatment a repeated measures analysis was used on the six times (6, 24, 30, 48, 54 and 72 h) with a residual option for the covariance structure (see Additional file 3 for a script of the mixed model parameters and options). Dunnett's test was used to compare each time point to the baseline value. When walking and landing data were compared between the control group and the ER-Bup group, mixed model analysis was used to test for the effect of treatment on the outcome measures in this crossover design.

Cat was included as a random effect in the mixed model (see Additional file 4 for a script of the mixed model parameters and options). Within each treatment a repeated measures analysis was used on the six times (6, 24, 30, 48, 54 and 72 h) with a residual option for the covariance structure. In this analysis, because of a significant period effect, each set of baseline data was separated and the average baseline value of each cat was calculated for each response measure. The difference between this average baseline value was subtracted from the measured response value at each time point in the analysis. Tukey's test was used to evaluate treatment effects at each time point. A Chi-square for independent test was used for NTf_1f_2. The values of $P < 0.05$ were considered significant.

Results

Pain score

No cats were administered rescue analgesia during study period. No pain scores of greater than two were recorded at any time for any cat (Fig. 2). For each cat, the area under the curve (AUC) of pain score above baseline versus time for both the control and ER-Bup treatment was calculated (Table 2). A paired t-test of AUC for control and ER-Bup group had a p-value of 0.0473, while a nonparametric test, Wilcoxon signed rank, gave the minimum possible value for four cats, namely 1/16 (0.063).

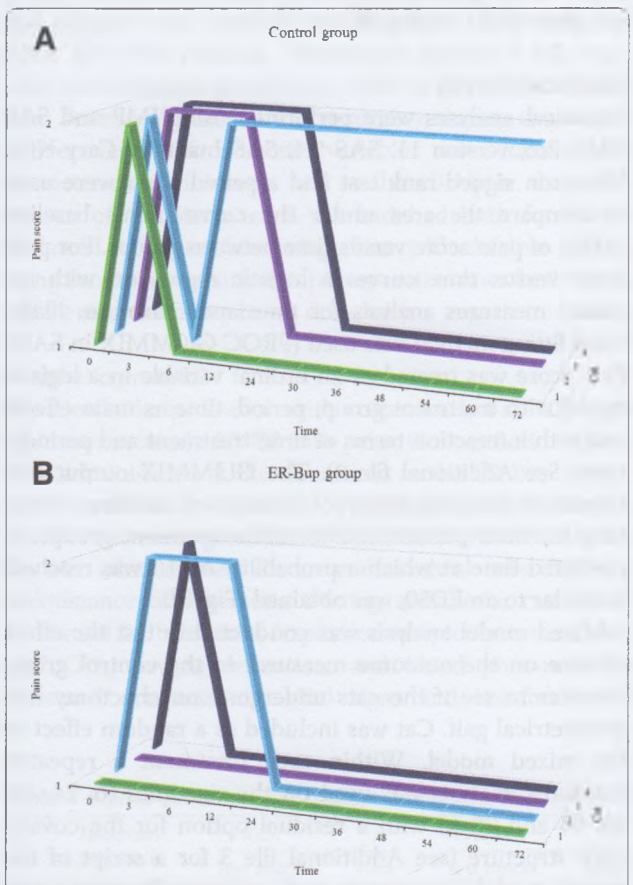

Fig. 2 Effect of the treatment on subjective pain score. 3D line graphs demonstrating the subjective pain scores for control (**a**) and extended release formulation of buprenorphine HCL (ER-Bup) groups (**b**) before and after onychectomy. Time point 0 h is the mean of assessments at 24 and 48 h prior to surgery (baseline). Observational pain assessments were performed at 3, 6, 12, 24, 30, 36, 48, 54, 60, and 72 h following surgery

The pain scores in Fig. 2 were further examined in a repeated measures logistic model with treatment group, period, and time as main effects and with interaction terms of time*treatment and period*time. Both time and period*time were significant factors (Table 3). The probability of the pain score being at baseline level versus time is shown in Fig. 3. The left panel is for the cats that received the control first and then ER-Bup, while the right panel is for the reverse sequence, ER-Bup then Control. For both panels the blue ER-Bup curves quickly rise toward a high probability of baseline score. The red control curves are both less than the ER-Bup curves and have quite different slopes. The horizontal reference line is a 0.50 probability of baseline score. Both ER-Bup curves reach the reference line before 6 h, while the left control curves has a crossing time around 55 h, while the right control curve crossed around 18 h. The large difference in the crossing times, similar to an LD-50, is the basis for the significant interaction term, period*time.

The cats were all successfully trained to walk across the PSW, and to jump down onto the PSW. Contemporaneous notes showed that the cats were jumping down onto the PSW after 3 days or training, but required the full 30 days allotted for training to consistently walk across the PSW in a straight line.

Landing data: comparison to baseline for control group

All cats satisfactorily completed five trials that could be analyzed through this study, although two baseline trials were missing due to technical errors. The outcome measures calculated for the landing data in control cats are summarized in Table 4 and Fig. 4, and time points that were significantly different from baseline are indicated. PVF and VI were significantly decreased for the operated limb and increased for the non-operated limb compared to baseline, and SI for PVF and for VI were significantly different from baseline in all time points. Tf_1f_2 and

Table 2 The area under the curve of pain score above baseline versus time

Cat	Control	ER-Bup
1	3	0
2	66	16.5
3	16.5	0
4	28.5	3

ER-Bup extended release formulation of buprenorphine HCL

Table 3 ANOVA table for repeated measures analysis of pain score versus time for PROC GLIMMIX

Effect	F value	Pr > F
Period	1.55	0.2686
Treatment	1.53	0.2714
Time	7.77	0.0069
Time*Treatment	1.14	0.2898
Time*Period	4.61	0.0353

Tf_1h_1 tended to be increased above baseline following surgery (Table 4).

NTf_1f_2 was 19 out of 78 trials (24%) in baseline period, and 59 out of 120 trials (49%) at following surgery period in control group ($P = 0.0045$, OR: 2.49).

Landing data: evaluation of treatment effect

The landing data is summarized in Tables 5 and 6 and Fig. 5. There was a significant treatment effect observed for all variables except for the Tf_1f_2. ER-Bup resulted in significantly greater PVF on the operated limb and significantly lower PVF on the non-operated limb compared to the control group. ER-Bup resulted in a significantly improved symmetry for PVF. ER-Bup also resulted in a significantly improved symmetry for VI. However, when compared to baseline, there was a greater drop in VI on the operated limb in the ER-Bup group, and a smaller increase in VI on the non-operated limb, compared to control. At first glance, these data are counter to the PVF data, but careful review of the data, including actual values, indicated that following surgery cats in the ER-Bup group had higher PVF values than the control group, but lower contact times (see Discussion). VI is the product of force multiplied by time. Although there was not a significant treatment effect on Tf_1f_2, values in the ER-Bup group were smaller, indicating less delay between the two limbs touching the mat compared to the control group. Tf_1h_1 (time between first forelimb and first hindlimb touching the mat) was longer in the control group than the ER-Bup group, indicating the hind limbs contacted the ground more quickly following the first contact of the fore limbs in the ER-Bup group compared to the control group.

NTf_1f_2 was 59 out of 120 trials (49%) in the control group, and 48 out of 120 trials (40%) in ER-Bup group. There was no significant difference between the two groups for NTf_1f_2 ($P = 0.15$).

Walking data: comparison to baseline for control group

The cats walked across the PSW at a faster velocity than the a priori target velocity, and so the comfortable speed

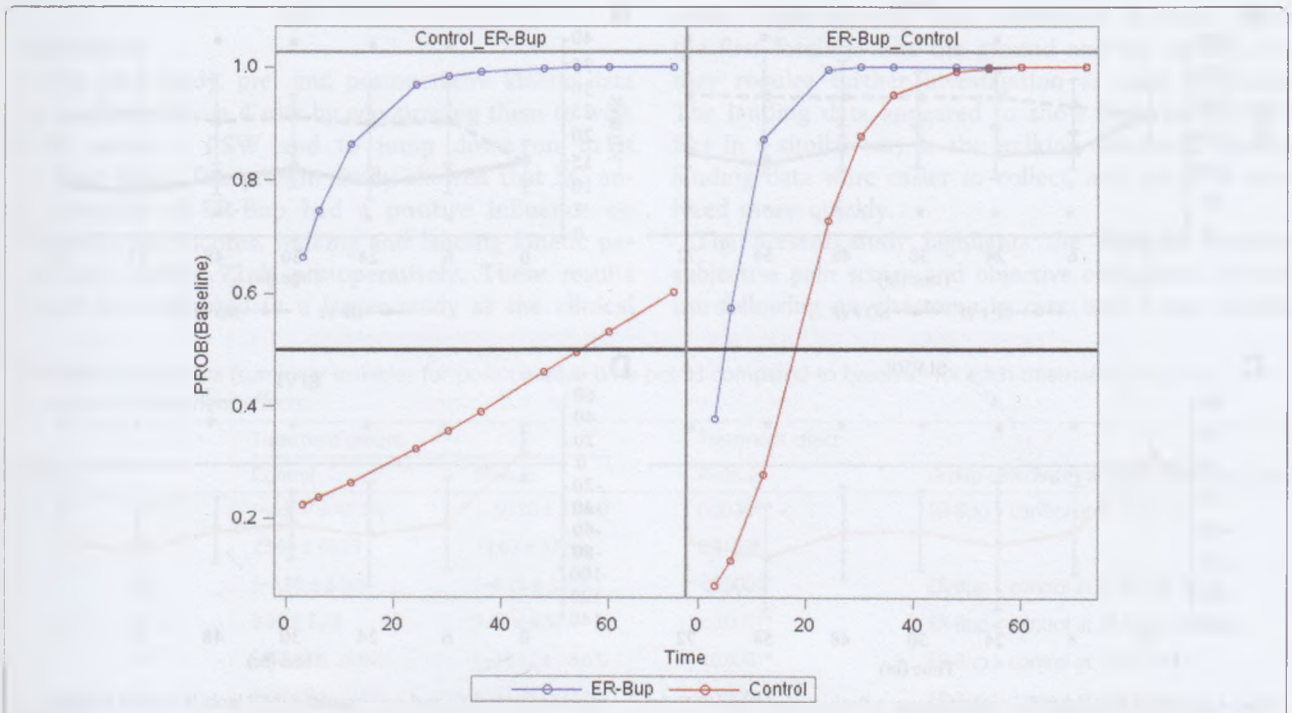

Fig. 3 Probability of pain score having a value equal to baseline versus time. The blue line represents the ER-Bup treatment and the red line represents control treatment. The left panel represents the sequence of saline, then ER-Bup injection, while right panel ER-Bup, then saline injection. Horizontal reference line at Probability = 0.5. The predicted time at which a probability of 0.5 is reached, similar to an LD50, is around the first time measurement (3 h) in the ER-Bup group (*blue curves*) in the left and right graphs. For the control group (*red curves*) the time for probability of 0.5 is quite different for the two periods, around 18 for the right graph and 55 for the left graph

Table 4 Kinetic data (mean ± SD) collected from control cats that had undergone a unilateral onychectomy and following landing from an elevated perch

Time	OP PVF (%)	NO PVF (%)	OP VI (%BW/sec)	NO VI (%BW/sec)	SI PVF	SI VI	Tf1f2 (sec)	Tf1h1 (sec)
P-value	<0.0001	0.0064	<0.0001	<0.0001	<0.0001	<0.0001	0.0125	<0.0001
0	189.14 ± 41.78	182.59 ± 52.18	15.02 ± 3.74	14.05 ± 5.30	4.86 ± 16.13	9.58 ± 24.58	(−0.0011 ± 0.012)	0.11 ± 0.024
6	129.07 ± 29.58*	197.07 ± 42.10	12.47 ± 3.76*	22.77 ± 8.27*	(−41.42 ± 28.22)*	(−52.93 ± 41.52)*	0.0060 ± 0.0094	0.15 ± 0.053*
24	123.98 ± 37.59*	208.88 ± 41.59*	10.54 ± 3.00*	21.99 ± 8.43*	(−51.01 ± 40.92)*	(−63.33 ± 46.62)*	0.0070 ± 0.0098	0.12 ± 0.014
30	138.75 ± 36.16*	210.07 ± 41.86*	13.27 ± 3.49	23.38 ± 5.44*	(−41.43 ± 25.83)*	(−54.12 ± 31.04)*	0.011 ± 0.010	0.14 ± 0.014*
48	132.88 ± 34.57*	209.55 ± 57.90*	11.51 ± 3.14*	22.79 ± 9.88*	(−43.13 ± 29.78)*	(−59.79 ± 30.42)*	0.012 ± 0.010	0.12 ± 0.020
54	105.87 ± 14.16*	209.88 ± 73.06*	9.04 ± 1.34*	24.72 ± 12.49*	(−59.72 ± 33.38)*	(−80.21 ± 39.60)*	0.018 ± 0.044*	0.12 ± 0.020
72	124.97 ± 23.18*	211.96 ± 58.16*	10.19 ± 1.87*	23.50 ± 9.91*	(−49.35 ± 26.43)*	(−70.77 ± 33.78)*	0.0090 ± 0.010	0.12 ± 0.020

*indicates significant difference from baseline (<0.05)
OP Operated limb, *NO* Non-operated limb, *PVF* Peak vertical force, *VI* vertical impulse, *SI* symmetry indices, *Tf1f2* Time between first and second forelimb strike, *Tf1h1* Time between first forelimb and first hindlimb strike

for traversing the PSW for each cat was considered acceptable. Postoperatively, some cats refused to walk across the PSW. In order to try to minimize the influence of velocity differences on the data, only SI was used for walking data analysis. Overall, cats traversed the walkway at 0.94 ± 0.41 (Mean ± SD) m/s in control group. At least three cats completed four to five trials that could be analyzed at each time point. One cat refused to walk at 6 h following surgery (control group) and another cat (control group) refused to walk except at 6 h after surgery in period 1. The summary values of SI for PVF and VI are shown in Table 7 and Fig. 6, and the time points where values were significantly different from baseline are indicated. SI for PVF and for VI were significantly reduced compared to baseline at all-time points.

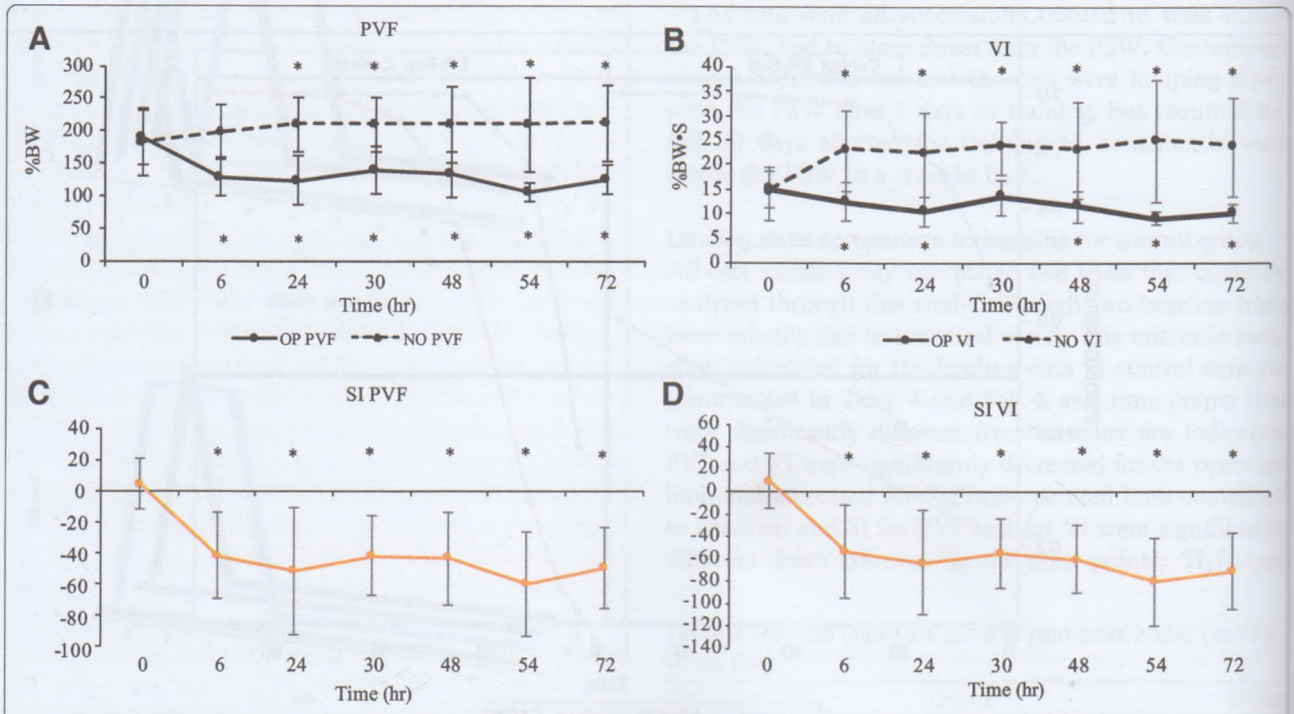

Fig. 4 Landing kinetic data for control group. **a** Peak vertical force (PVF) for the operated limb (OP) and non-operated (NO) limb; **b** Vertical impulse (VI) for OP and NO; **c** Symmetry indices (SI) for PVF; **d** SI for VI. Line graphs demonstrating the mean ± SD in control group at various time points before and after onychectomy ($n = 4$). Solid line is OP, dotted line is NO. Time point 0 h is the mean of each gait parameter at 24 and 48 h prior to surgery (baseline). The gait parameters were recorded at 6, 24, 30, 48, 54, and 72 h following surgery. The SI of 0 means there is perfect symmetry between the forelimbs, the value of −200 means the cat is non-weight bearing on the operated limb. * indicates significant difference from baseline in each parameter, shown above upper line and below lower line (<0.05)

Table 5 Kinetic data (mean ± SD) collected from ER-Bup treated cats that had undergone a unilateral onychectomy and following landing from an elevated

Time	OP PVF (%)	NO PVF (%)	OP VI (%BW/sec)	NO VI (%BW/sec)	SI PVF	SI VI	Tf1f2 (sec)	Tf1h1 (sec)
P-value	<0.0001	0.3800	<0.0001	0.0004	<0.0001	<0.0001	0.0002	<0.0001
0	189.57 ± 52.03	173.68 ± 43.23	16.86 ± 5.82	14.75 ± 5.18	7.95 ± 20.60	14.15 ± 31.55	(−0.0005 ± 0.0071)	0.12 ± 0.027
6	156.33 ± 45.68*	180.15 ± 28.05	11.59 ± 3.53*	13.61 ± 2.83	(−17.02 ± 22.82)*	(−17.54 ± 34.80)*	0.0090 ± 0.012*	0.11 ± 0.020
24	132.74 ± 48.23*	177.95 ± 37.77	10.80 ± 3.45*	18.34 ± 6.15*	(−32.71 ± 28.98)*	(−49.02 ± 42.24)*	0.0040 ± 0.0082	0.11 ± 0.017
30	146.57 ± 35.39*	195.21 ± 51.45	11.79 ± 2.45*	17.76 ± 5.46	(−28.07 ± 25.06)*	(−36.69 ± 34.91)*	0.007 ± 0.0098*	0.12 ± 0.025
48	128.38 ± 36.61*	186.89 ± 31.34	9.91 ± 3.01*	15.55 ± 3.61	(−38.66 ± 28.26)*	(−44.44 ± 37.28)*	0.007 ± 0.0098*	0.10 ± 0.026*
54	136.68 ± 46.94*	183.54 ± 52.03	10.45 ± 2.37*	15.95 ± 5.03	(−30.78 ± 29.88)*	(−30.78 ± 29.88)*	0.008 ± 0.010*	0.095 ± 0.017*
72	133.76 ± 47.14*	188.15 ± 28.06	9.12 ± 1.99*	18.49 ± 7.84*	(−37.39 ± 32.35)*	(−59.57 ± 47.28)*	0.011 ± 0.010*	0.097 ± 0.028*

Mean ± SD, *indicates significant difference from baseline (<0.05)
OP Operated limb, *NO* Non-operated limb, *PVF* Peak vertical force, *VI* vertical impulse, *SI* symmetry indices, *Tf1f2* Time between first and second forelimb strike, *Tf1h1* Time between first forelimb and first hindlimb strike

Walking data: evaluation of treatment effect

No cats in ER-Bup group refused to walk across the PSW. Cats in ER-Bup group traversed the walkway at 1.16 ± 0.36 m/s, which was significantly faster than the control group ($p < 0.0001$). The summary values of SI for PVF and VI are shown in Table 8 and Fig. 7, and the time points where values were significantly different between groups are indicated. SI for PVF and VI were significantly different between the groups, indicating significantly greater symmetry for these variables in the ER-Bup group.

Discussion

In this pilot study, pre- and postoperative kinetic data were collected from 4 cats by encouraging them to walk freely across a PSW and to jump down on to it (landing data). The present study showed that SC administration of ER-Bup had a positive influence on subjective pain scores, walking and landing kinetic parameters, during 72 h postoperatively. These results should be confirmed in a larger study as the clinical significance of our results is difficult to judge in a small pilot study.

To the authors' knowledge this is the first report that has used jumping down onto a PSW for evaluation of limb use following surgery. Landing data from cats that had undergone onychectomy could be collected from all cats at all-time points, unlike walking data. In addition, the landing data appeared to provide desired information, and additionally other outcome measures could be collected, such as Tf_1f_2, Tf_1h_1, and NTf_1f_2. These are novel outcome measures that intuitively make sense, especially the time difference between when the first forelimb hits the ground and the second, but they require further investigation as valid measures. The landing data appeared to show the treatment effect in a similar way to the walking data. Additionally, landing data were easier to collect, and could be analyzed more quickly.

The present study highlights the disparity between subjective pain scores and objective evaluations of limb use following onychectomy in cats, and if one accepts

Table 6 Landing data (summary variables for postoperative time points compared to baseline) for each treatment group and tabluation of treatment effects

		Treatment groups		Treatment effect	
Variable		Control	ER-Bup	*P*-values	Group differences at individual time points
PVF	OP	(−63.97 ± 45.59)	(−50.50 ± 37.44)	0.0026*	ER-Bup > control at 6, 54 h
	NO	23.69 ± 40.23	11.63 ± 37.58	0.0169*	
VI	OP	(−3.89 ± 3.50)	(−6.25 ± 3.22)	<0.0001*	ER-Bup < control at 6, 30, 48, 72 h
	NO	8.99 ± 7.23	1.87 ± 4.50	<0.0001*	ER-Bup < control at all time points
SI	PVF	(−52.03 ± 26.02)	(−38.72 ± 18.63)	<0.0001*	ER-Bup > control at 6, 24, 54 h
	VI	(−72.28 ± 29.47)	(−54.96 ± 27.10)	<0.0001*	ER-Bup > control at 6, 30, 54 h
Tf1f2		0.012 ± 0.021	0.008 ± 0.010	0.076	
Tf1h1		0.014 ± 0.029	(−0.015 ± 0.023)	<0.0001*	ER-Bup < control at all time points

Mean ± SD, *indicates significant difference between groups (<0.05)
OP Operated limb, *NO* Non-operated limb, *PVF* Peak vertical force, *VI* vertical impulse, *SI* symmetry indices, *Tf1f2* Time between first and second forelimb strike, *Tf1h1* Time between first forelimb and first hindlimb strike, *ER-Bup* extended release formulation of buprenorphine HCL

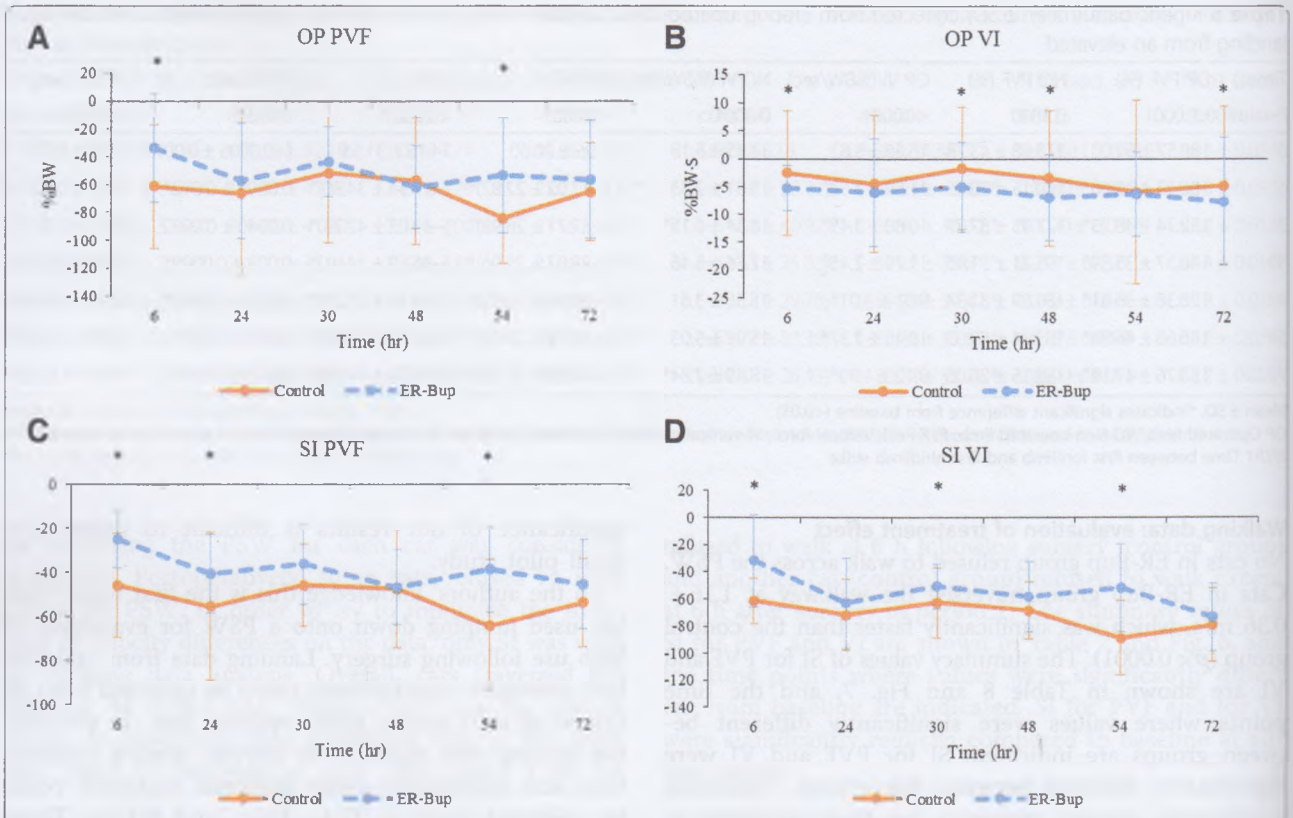

Fig. 5 Effect of treatment with ER-Bup on landing kinetic data. **a** Peak vertical force (PVF) for operated limb (OP); **b** Vertical impulse (VI) for OP; **c** Symmetry indices (SI) for PVF; **d** SI for VI. Assessments were made 24 and 48 h initial and second surgery (baseline) and 6, 24, 30, 48, 54, and 72 h after surgery. The average baseline value was subtracted from the measured value at each time point in the analysis. Line graphs showing the changes from baseline in each gait parameter over study period. The mean difference ± SD in control group and extended release formulation of buprenorphine HCL (ER-Bup) group at various time points after onychectomy ($n = 4$). Orange solid line is control group, blue dotted line is ER-Bup group. Negative value of SI means the cat put less weight on operated limb after surgery.* indicates significant difference between groups (<0.05)

that in this situation decreased limb use is an indicator of pain, our data clearly point to the need for development of more sensitive, and valid, subjective measures. Based on our subjective pain scores assigned by trained observers, no cats required rescue analgesics in either group, and the cats looked comfortable at the end of study. No cat scored greater than two out of five at any time during the study, and the direct experience of PDK. DB and BDXL in these studies supports the result that the cats looked comfortable. That is not to say we believe these cats are comfortable, on the contrary, our kinetic data showed large asymmetry in kinetic variables between the operated and non-operated limbs suggesting discomfort associated with the procedure. However, this is very difficult to detect subjectively.

Table 7 Kinetic data collected during walking in the control group

Time	SI PVF	SI VI
P-value	<0.0001	<0.0001
0	2.20 ± 13.43	4.54 ± 12.17
6	(−73.00 ± 49.92)*	(−112.32 ± 43.53)*
24	(−25.39 ± 20.54)*	(−40.87 ± 24.84)*
30	(−41.43 ± 25.83)*	(−54.12 ± 31.04)*
48	(−31.69 ± 21.16)*	(−59.40 ± 31.47)*
54	(−52.48 ± 44.68)*	(−80.71 ± 54.10)*
72	(−31.82 ± 19.04)*	(−65.90 ± 31.35)*

Mean ± SD, *indicates significant difference from baseline (<0.05)

A previous study has suggested an analgesic effect of ER-Bup, however the pain evaluations consisted of two non-validated subjective pain scales [23] and no placebo group. In contrast, our study showed a clear analgesic effect over a 72 h period postoperatively using objective measures and a control group comparison.

However, a weakness of our study is that the subjective scoring system used was not one of the partially validated subjective pain scales currently available [14, 16–18]. This was because the study was performed before most of the information on these scales was published. Additionally, the assessment tools that have been produced thus far

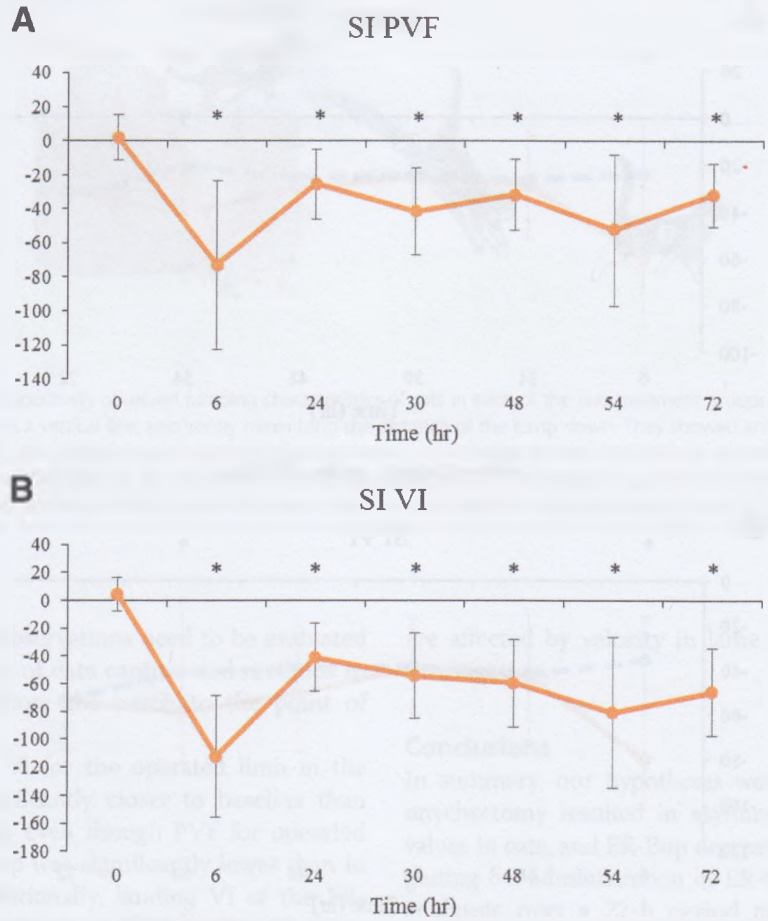

Fig. 6 Summary symmetry indices values for kinetic data in the control group during walking. **a** Symmetry indices (SI) for Peak vertical force (PVF); **b** SI for Vertical impulse (VI). Line graphs showing the mean ± SD in control group at various time points before and after onychectomy in four walking cats. Time point 0 h is the mean of assessments at 24 and 48 h prior to surgery (baseline). The gait parameters were recorded at 6, 24, 30, 48, 54, and 72 h following surgery. The SI of 0 means there is perfect symmetry between the forelimbs, the value of −200 means the cat is non-weight bearing on the operated limb. *indicates significant difference from baseline (<0.05)

have been developed for client-owned cats, not research cats. Although there is no data on this subject, our observations are that research cats are less demonstrative than client-owned cats and other unpublished observations indicate these subjective tools are less sensitive in research cats than client-owned cats, similar to the situation in dogs [22].

Kinetic parameters measured using a PSW in cats during walking have been used to evaluate the effectiveness of analgesics for acute and chronic pain [21, 26, 27]. However, although several reports have evaluated gait symmetry and limb loading in cats, some of them have accepted the data generated even if a limb only contacted the walkway once or twice [26, 28] and wide ranges of velocity are accepted [19]. The present study highlighted the difficulty in collecting walking data from freely moving cats as velocity cannot be easily controlled, and in some cases, cats in the saline treatment

Table 8 Kinetic data (mean ± SD) collected during walking in the control and ER-Bup groups

	Treatment groups		Treatment effect	
Variable	Control	ER-Bup	*P*-values	Group difference in time
SI for PVF	(−54.61 ± 27.98)	(−41.57 ± 22.03)	0.0002*	ER-Bup > control at 6, 54 h
SI for VI	(−36.33 ± 25.59)	(−26.13 ± 21.89)	<.0001*	ER-Bup > control at 6, 54 h

*Indicates significant difference between groups (<0.05)
PVF Peak vertical force, *VI* vertical impulse, *SI* symmetry indices, *ER-Bup* extended release formulation of buprenorphine HCL

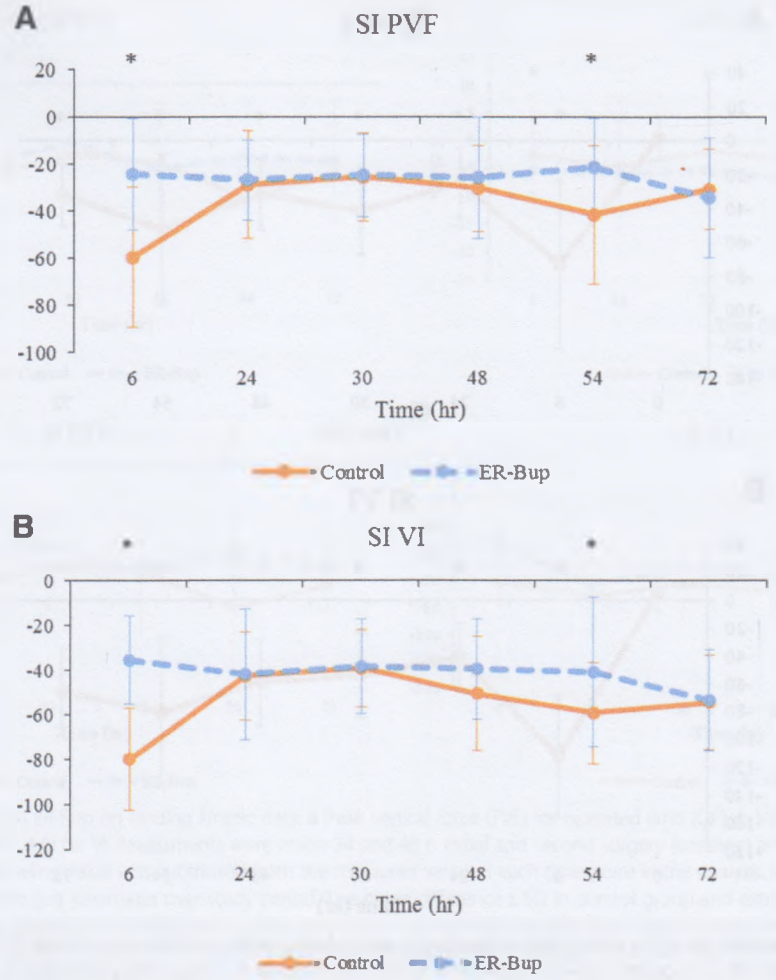

Fig. 7 Effect of the treatment on symmetry indices values for kinetic data during walking. **a** Symmetry indices (SI) for Peak vertical force (PVF); **b** SI for Vertical impulse (VI). Assessments were made 24 and 48 h initial and second surgery (baseline) and 6, 24, 30, 48, 54, and 72 h after surgery. The difference between this average baseline value and the measured value were calculated at each time point in the analysis. Line graphs showing the mean difference ± SD in control group and extended release formulation of buprenorphine HCL (ER-Bup) group at various time points after onychectomy ($n = 4$). Orange solid line is control group, blue dotted line is ER-Bup group. Negative value of SI means the cat put less weight on operated limb after surgery.* indicates significant difference between groups (<0.05)

group refused to walk. Variation in velocity is a well-known factor that influences the kinetic data during walking [29, 30]. Conversely, landing data are easily collected, although this approach is not likely to be useful to assess hindlimb pain.

Although there were significant differences between baseline and postoperative period in Tf_1f_2 and NTf_1f_2, significant differences were not observed between the two treatment groups. The coarseness of data collection may have contributed to the lack of difference. The frequency of data acquisition was limited to 60 Hz using the PSW and so a single frame is equivalent to 0.017 s. This frequency of data acquisition might be not fast enough to detect changes in the time between first forelimb and second forelimb strike.

A significant increase in Tf_1h_1 was detected after surgery and values were significantly longer in the control group. At first glance, this is counter intuitive – one would expect that a cat landing from a jump, and with a painful forelimb, would more quickly bring the hindlimbs down to the ground. In order to try to explain this, we retrospectively evaluated the videos captured during the study, and we observed that cats in the control group appeared to jump down more vertically, appearing to be trying to shorten the jump distance, but conversely, appeared to spend longer on their forelimbs following jumping. Cats in the ER-Bup group tended to jump more normally, jumping out, away from the platform, and thus the hindlimbs made contact with the PSW more quickly. These observations are detailed in

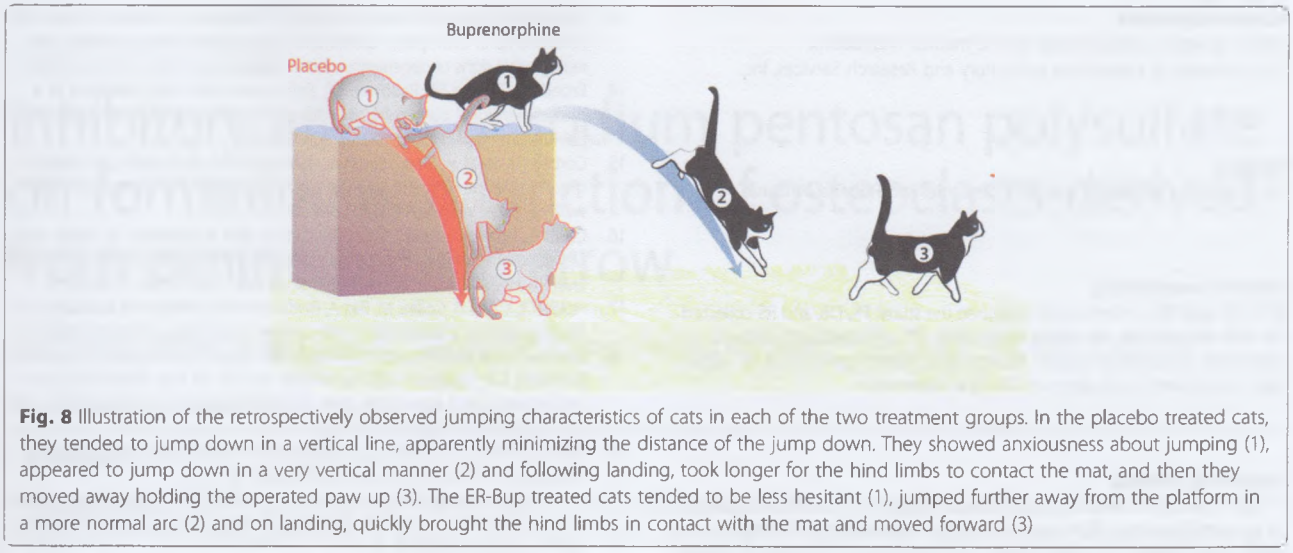

Fig. 8 Illustration of the retrospectively observed jumping characteristics of cats in each of the two treatment groups. In the placebo treated cats, they tended to jump down in a vertical line, apparently minimizing the distance of the jump down. They showed anxiousness about jumping (1), appeared to jump down in a very vertical manner (2) and following landing, took longer for the hind limbs to contact the mat, and then they moved away holding the operated paw up (3). The ER-Bup treated cats tended to be less hesitant (1), jumped further away from the platform in a more normal arc (2) and on landing, quickly brought the hind limbs in contact with the mat and moved forward (3)

Fig. 8. However, these, observations need to be evaluated using a higher frequency of data capture and methods to measure the distance from the perch to the point of landing on the PSW.

Surprisingly, landing VI for the operated limb in the control group was significantly closer to baseline than VI in the ER-Bup group even though PVF for operated limb in the control group was significantly lower than in the ER-Bup group. Additionally, landing VI of the ER-Bup group was significantly lower than landing VI of the control group. Lower VI during walking and trotting in dogs has been associated with pain and lameness. Our apparently contradictory results for the landing data appears to be due to the contact time. The contact time for operated limb in the control group was longer than in the ER-Bup group (see Additional files 5 and 6). Vertical impulse is defined as the area under the force by time curve. Thus, if contact time is longer, VI may be larger than in another case where force is higher but contact time is shorter. All our other data indicates a positive benefit from the administration of ER-Bup, and therefore we hypothesize that the reason for the longer contact time for operated limb in the control group may be related to a similar phenomenon in humans – humans prefer lower pain for longer, than higher pain for shorter time periods [31]. As described above, the way the cats chose to jump down from the elevated platform may help explain our data.

Limitations of this study include the very small number of the cats evaluated, and the variations in velocity during walking. The small sample size limits statistical power. To minimize the effect of velocity on PVF and VI, velocity should be restricted to a tight range [29, 30]. We used SI as a means of diminishing the effect of velocity, however, it is not known if symmetry indices are affected by velocity in lame animals and this needs investigation.

Conclusions

In summary, our hypotheses were supported. Unilateral onychectomy resulted in asymmetry of forelimb kinetic values in cats, and ER-Bup decreased this asymmetry, suggesting SC administration of ER-Bup may be an effective analgesic over a 72-h period postoperatively. Further, data indicate jumping down onto a PSW may be a useful and efficient way of assessing analgesics in cats if a unilateral model of limb pain is used. Further studies are needed to extend our understanding of landing kinetic data in cats.

Additional files

Additional file 1: Subjective pain scoring system used.

Additional file 2: Script of PROC GLIMMIX.

Additional file 3: Script of the mixed model parameters and options.

Additional file 4: Script of the mixed model parameters and options.

Additional file 5: Contact time for operated and non-operated limbs for each group.

Additional file 6: Relationship between contact time and Peak vertical force (PVF) in operated limb (OP).

Abbreviations

ER-Bup: Extended release formulation of buprenorphine HCL; NTf_1f_2: Number of trials where there was a measurable difference time of first and time of second forelimb strike; PSW: Pressure sensitive walkway; PVF: Peak vertical force; SI: Symmetry indices; Tf_1f_2: Time between first and second forelimb strike; Tf_1h_1: Time between first forelimb and first hindlimb strike; VI: Vertical impulse

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Authors' contributions
BDXL, PK and DB conceived and designed the study; PK, DB, and RS collected the data; JH extracted the kinetic values from the PSW output; CS and ME performed the statistical analysis; ME and BDXL drafted manuscript; all authors read, contributed to the approved the final manuscript.

Competing interests
P. Kigin is employed and D. Bledsoe was employed at the time of the research by Farnam Companies. BDX Lascelles was a paid consultant in the initial study ideation and design. The authors have no other conflicts of interest to declare.

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Inhibitory effects of sodium pentosan polysulfate on formation and function of osteoclasts derived from canine bone marrow

H. M. Suranji Wijekoon*, Eugene C. Bwalya, Jing Fang, Sangho Kim, Kenji Hosoya and Masahiro Okumura

Abstract

Background: Sodium pentosan polysulfate (NaPPS) was testified as a chondroprotective drug in with a detailed rationale of the disease-modifying activity. This study was undertaken to determine whether anti-osteoarthritis drug, NaPPS inhibited osteoclasts (OC) differentiation and function. Canine bone marrow mononuclear cells ($n = 6$) were differentiated to OC by maintaining with receptor activator of nuclear factor kappa B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) for up to 7 days with the treatment of NaPPS at concentration of 0, 0.2, 1 and 5 μg/mL. Differentiation and function of OC were accessed using tartrate-resistant acid phosphate (TRAP) staining and bone resorption assay, while monitoring actin ring formation. Invasion and colocalization patterns of fluorescence-labeled NaPPS with transcribed gene in OC were monitored. Gene expression of OC for cathepsin K (CTK), matrix metallopeptidase-9 (MMP-9), nuclear factor of activated T-cells cytoplasmic 1 (NFATc1), c-Fos, activator protein-1(AP-1) and carbonic anhydrase II was examined using real-time PCR.

Results: Significant inhibition of OC differentiation was evident at NaPPS concentration of 1 and 5 μg/mL ($p < 0.05$). In the presence of 0.2 to 5 μg/mL NaPPS, bone resorption was attenuated ($p < 0.05$), while 1 and 5 μg/mL NaPPS achieved significant reduction of actin ring formation. Intriguingly, fluorescence-labeled NaPPS invaded in to cytoplasm and nucleus while colocalizing with actively transcribed gene. Gene expression of CTK, MMP-9 and NFATc1 were significantly inhibited at 1 and 5 μg/mL ($p < 0.05$) of NaPPS whereas inhibition of c-Fos and AP-1 was identified only at concentration of 5 μg/mL ($p < 0.05$).

Conclusions: Taken together, all the results suggest that NaPPS is a novel inhibitor of RANKL and M-CSF-induced CTK, MMP-9, NFATc1, c-Fos, AP-1 upregulation, OC differentiation and bone resorption which might be a beneficial for treatment of inflammatory joint diseases and other bone diseases associated with excessive bone resorption.

Keywords: Sodium pentosan polysulfate, Osteoclast differentiation, Bone marrow, Bone resorption, Dog

Background

Bone homeostasis is crucial to maintain the integrity of bone functions that coordinates balance between bone resorption by osteoclasts (OC) and bone formation by chondrocytes/osteoblasts [1, 2]. It is well established that an imbalance in the function of OC and osteoblasts has severe consequences for the organism, leading to serious bone pathologies such as osteoporosis, joint and bone diseases involving the immune system including, rheumatoid arthritis (RA) and periodontal disease [3]. Osteoclasts, which originate from monocyte/macrophage lineage from bone marrow hematopoietic precursors [4] are the principal multinucleate giant resorptive cells of bone. Two major factors such as macrophage colony-stimulating factor (M-CSF) and receptor activator of NF kappa B ligand and (RANKL) are required for their differentiation and maturation [5–7]. Receptor activator of NF kappa B ligand binds to its receptor, receptor activator of NF kappa B on

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OC precursors [8], which involve the activation of NF-kB and Jun N-terminal kinase, eventually leading to the expression of activator protein-1 (AP1)/ Fos, an essential regulator of OC differentiation [9]. Previously reports have shown that OC precursors are found in both peripheral blood and synovial tissues of human with RA [10, 11]. Osteoclast precursors which are unable to resorb bone [12] stained positive for tartrate-resistant acid phosphatase (TRAP) eventually fuse to form multinucleated mature TRAP-positive OC [13]. Thus maturation and functional capability of OC while differentiation, are critical cellular process, understanding its regulation will have an important impact on the development of a new therapy to control bone loss among human and dogs.

In the past few years, the concept of disease -modifying osteoarthritis drugs (DMOADs) have been explored as an alternative treatment modality for osteoarthritis (OA) [14] instead of using nonsteroidal anti-inflammatory drugs (NSAIDs) which has been frequently used for treating OA and associate with a high risk for gastrointestinal lesions with long-term uses [15]. Sodium pentosan polysulfate (NaPPS) is a semi-synthetic sulfated polysaccharide drug manufactured from European beechwood hemicellulose by sulfate esterification with the average molecular weight of 5700 Da [16]. From the results of previous in vitro and in vivo studies, the spectrum of pharmacological activities exhibited by NaPPS would qualify it as DMOADs [17] because of its ability to preserve the integrity of the articular cartilage and bone while improving the quality of the joint synovial fluid [18–21].

Although PPS has been used for a number of years for the treatment of thrombotic and hyperlipidemic indications [20] it has only recently been shown to be effective in improving the symptoms of human patients with OA [16]. While the molecular mechanism of PPS action at the microenvironment of joint remains unclear, some previous reports show that, NaPPS is capable in enhancing synthesis of proteoglycans such as aggrecan, which is intimately associated with resist compression throughout the extracellular matrix of articular cartilage [22]. Hence, synovial changes in dogs with canine arthritis mimic human RA, dogs are the potential useful model for studies of therapy [23]. Recently, it has been reported that NaPPS can inhibit osteogenic differentiation in human bone marrow derived precursor cells while inducing chondrogenic differentiation from bone marrow-derived mesenchymal stem cells in canine as well as human [24, 25]. Use of progenitor cells origin from different sources is being used for investigating the therapeutic effects and to imply its clinical uses [26]. However, among the several previous human and animal studies of NaPPS based on cartilage research, there was dearth of information on effect of NaPPS over the osteoclastogenesis, anti-resorptive capability and influence on cell signaling molecules of OC. To the best of our knowledge, present study is the first attempt to identify the interaction of NaPPS with in vitro cultured OC-derived from dog bone marrow. The objective of the study reported here was to determine whether there was an effect of NaPPS on osteoclastogenesis of canine bone marrow-derived hematopoietic precursor cells. We hypothesized that the NaPPS, which carry different effect by improving the symptoms of OA would more likely to have an inhibitory effect on OC differentiation and its signaling pathways.

Methods

Osteoclastic differentiation from canine bone marrow

Proximal femur of one year old, healthy beagle dogs (n = 6) were used to collect the 5 mL of bone marrow samples in to 10 mL syringe containing 1 mL Dulbecco's modified eagle's medium (DMEM, Life technologies, New York, USA) and 1000 U/mL of heparin (Nipro, Osaka, Japan). The use of all samples from healthy experimental dogs was in accordance with Hokkaido University Institutional Animal Care and Use Committee guidelines (approval number: 12–0059). Separation of bone marrow mononuclear cell (BMMs) fraction was done and preceded as described previously [27, 28]. Briefly, BMMs was obtained by density gradients centrifugation over lymphoprep (Axis-sheild PoC AS, Oslo, Norway) to remove red blood cells. Isolated BMMs cell fraction (5×10^6 cells/mL) was incubated with DMEM containing penicillin/streptomycin (100 units/mL, Wako pure chemical, Tokyo, Japan) and 10% heat-inactivated fetal bovine serum (FBS, Nichirei Bioscience INC., Tokyo, Japan) for 24 h to separate the non-adherent and adherent cells. Non-adherent were collected as a source of immature OC precursors, suspended in DMEM, counted, seeded on 48-well plates (Corning, New York, USA) at 2×10^5 cells/well, and cultured in DMEM with the presence of 20 ng/ml recombinant human M-CSF (Invitrogen, Maryland, USA) for 3 days. After 3 days, adherent cells were used as OC precursors after washing out the non-adherent cells, including lymphocytes and further cultured in the presence of 25 ng/mL M-CSF, 50 ng/mL recombinant human RANKL (Sigma-Aldrich, St Louis, Missouri, USA) to generate osteoclast-like multinucleated giant cells. The cells were treated with 0, 0.2, 1 and 5 μg/mL concentration of NaPPS (Cartrophen Vet-Biopharm-100 mg/ml, New South Wales, Australia) for 1-week. The selected concentrations of NaPPS are within the previous proved non-cytotoxic range for bone marrow derived cells [24]. Triplicate cultures for each concentration of NaPPS were maintained by changing the media in every 48 h ensuring their constancy of concentrations.

Tartrate-resistant acid phosphate (TRAP) staining

Cultured BMMs with M-CSF and RANKL in the presence or absence of NaPPS were subjected to TRAP stain (Cosmo Bio Co., LTD, Tokyo, Japan) after 7 days. Cells were washed with 1% phosphate buffered saline (PBS) and fixed with 10% formalin neutral buffer solution for 5 min at room temperature. After washing with 500 µL deionized water 3 times, cells were stained for TRAP according to the manufacturer's instructions. Cells containing ≥3 nuclei were considered as OC and counted.

Pit formation assay

Non-adherent cells, collected from BMMs fraction of 3 dogs were cultured at 2×10^5 cells/well density on bone resorption assay plate 48 (PG Research, Tokyo, Japan) which was coated with calcium phosphate (CaP-coated). The cells were maintained in DMEM with the presence of 20 ng/ml recombinant human M-CSF (Invitrogen, Maryland, USA) for 3 days in triplicate cultures. After 3 days, adherent cells were used as OC precursors after washing out the non-adherent cells and further cultured in the presence of 25 ng/mL M-CSF, 50 ng/mL recombinant human RANKL and NaPPS at various concentrations (0, 0.2, 1 and 5 µg/mL). After 7 days, the CaP-coated plate was treated with 5% sodium hypochlorite (Sigma-Aldrich, St Louis, Missouri, USA) for 5 min according to the manufacturer's instructions. The resorption pit area was analyzed and counted by Image-J software (Image J software version 1.43, National Institute of Health).

Actin ring formation assay

The actin ring formation assay was performed as described previously [29]. Briefly, BMMs cultured with M-CSF, RANKL and various concentrations of NaPPS for 7 days were washed with PBS and fixed with 4% paraformaldehyde (Wako pure chemical, Tokyo, Japan) in PBS on ice for 20 min. Osteoclasts were detergent-permeabilized with 0.2% Triton X-100 (ICN Biomedicals, Germany) in PBS for 10 min, washed and blocked in 10% normal goat serum (Sigma-Aldrich, St Louis, Missouri, USA) in PBS for 1 h. The cells were incubated with primary rabbit anti-F actin polyclonal antibody (Bioss Inc., Massachusetts, USA) (1:100 dilution) for 1 h in PBS with 1% normal goat serum, washing three times with PBS, incubating for 1 h with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (Sigma) (1:100 dilution) in PBS with 1% normal goat serum, washing three times with PBS, and finally mounting with aqueous mounting medium. The images were observed by counting the number of actin rings under a laser scanning confocal microscope (Zeiss, Illinois, USA).

Immunocytochemical detection of localization of NaPPS with actively transcribed gene

Osteoclast precursors resulting from canine bone marrow cells (2×10^5 cells) were cultured in 8-well culture slide (Iwaki, Tokyo, Japan) in 400 µL of DMEM, 10% FBS with OC differentiation factors. Cells were incubated with 10 µg/mL of Tetramethylrhodamine (TRITC)-labeled NaPPS (Arthropharm, New South Wales, Australia) for 24 h. After fixation and blocking, cells were incubated with primary anti-human c-Jun (HT-9) rabbit polyclonal antibody (Santa Cruz Biotechnology, Dallas, Texas, USA) (1:100 dilution) in 1% normal goat serum followed by incubation with FITC-conjugated goat anti-rabbit antibody (Sigma) (1:100 dilution) in 1% normal goat serum. The OC were observed for detecting the colocalization patterns of NaPPS with transcribed gene (c-Jun) under a laser scanning confocal microscope.

mRNA isolation and RT-PCR

Total RNA from cells was extracted using RNeasy Mini Kit (QIAGEN, Germantown, Maryland, USA) according to the manufacture's protocol. Total RNA was quantified by spectrophotometry at 260 nm. RNA with a 260/280 nm ratio in the range 1.8–2.0 was considered high quality and then transcribed into cDNA with M-MLV RT kit (Takara Bio, Tokyo, Japan) according to manufacturer's recommended procedures. One microgram of total RNA derived was reverse-transcribed into cDNA with random hexamers. PCR conditions were as follows: denaturation at 95 °C for 30 s, annealing temperature for 1 min, extension at 72 °C for 1 min for 30 cycles, and final extension at 72 °C for 7 min. PCR products were separated on 1.5% agarose gel (BM Equipment, Tokyo, Japan) and stained with ethidium bromide (Nippon Gene, Tokyo, Japan).

Real-time PCR

Quantitative real-time PCR analysis was performed with KAPA SYBR® FAST qPCR kit (KAPA). The amount of 2 µL of cDNA template was added to each 10 µL of premixture with specific primers. The following primer sets were used: carbonic anhydrase II (CAII), 5′-AAGG AGCCCATCAGCGTTAG-3′ (forward) and 5′-GGGCG CCAGTTATCCATCAT-3′ (reverse); NFATc1, 5′-CAC AGGCAAGACTGTCTCCA-3′ (forward) and 5′-TCCT CCCAATGTCTGTCTCC-3′ (reverse); MMP-9, 5′-GG CAAATTCCAGACCTTTGA-3′ (forward) and 5′-TAC ACGCGAGTGAAGGTGAG-3′ (reverse); c-Fos, 5′- GT CCGTACAGACCACAGACC-3′ (forward) and 5′-CGC TCCACTTCATTGTGCTG-3′ (reverse); CTK, 5′- ACC CATATGTGGGACAGGAT-3′ (forward) and 5′-TGGA AAGAGGTCAGGCTTGC-3′ (reverse); AP-1, 5′-TCTA CGACGATGCCCTCAAC-3′ (forward) and 5′-TGAGCA GGTCCGAGTTCTTG-3′ (reverse); GAPGH, 5′-CTGA ACGGGAAGCTCACTGG-3′ (forward) and 5′-CGATG

CCTGCTTCACTACCT-3′ (reverse). All reactions were normalized to the housekeeping gene b-Actin. glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistical analysis

Quantitative real-time PCR data, number of OC, resorption pits and actin rings were analyzed using SPSS software (SPSS software ver. 07 for Windows; SPSS Inc., Chicago, Illinois, USA). Analysis of variance (ANOVA) was used to compare the mean values between the treatments. Where significant different observed, multiple comparison of group means was performed using Post Hoc Bonferroni. Significant level was defined as $p < 0.05$. All quantitative results are presented as mean ± SE.

Results

The effect of different concentration of NaPPS on OC differentiation from BMMs stimulated with RANKL and M-CSF was evaluated. The number of TRAP-positive multinuclear cells (≥3 nuclei) generated in 48 well plate were reduced with the administration of NaPPS at the concentration of 1 and 5 μg/mL NaPPS ($p < 0.05$) (Fig. 1a, b). Osteoclast specific genes, CAII, CTK and MMP-9 expression were analyzed after treatment of NaPPS at the concentration of 0.2, 1and 5 μg/mL. Relative mRNA expression level of CTK and MMP-9 genes were significantly downregulated ($p < 0.05$) at 1 and 5 μg/mL concentrations of NaPPS (Fig. 1c, d, e).

NaPPS on bone resorption was assessed with OC generated from 3 dogs. Cells were plated on CaP-coated plates and stimulated with M-CSF and RANKL in the presence or absence of NaPPS. Cells stimulated with M-CSF and RANKL formed a number of resorption pits (Fig. 2a, b), suggesting that the bone resorption activity of RANKL-treated cells made them into functionally active state resembling OC. All the concentrations of NaPPS (0.2, 1 and 5 μg/mL) significantly reduced the formation of resorption pits in number and in overall area compared with treatment with M-CSF and RANKL alone. In the presence of RANKL exposure, BMMs can differentiate into mature OC and form distinct actin-ring structures (Fig. 2c, d). However, NaPPS significantly reduced the number of actin-ring structures at 1 and 5 μg/mL concentrations, suggesting the inhibitory effect of certain concentration of NaPPS over the functional unit of OC.

Canine OC were treated with fluorescently labelled (red) pentosan polysulfate (TRITC-PPS), then fixed in formalin and fluorescently stained for genetic DNA (blue) and for c-Jun (green) which is one of the key components of AP-1 transcription factor [12]. Fluorescence-labeled NaPPS invaded in to cytoplasm and nucleus (Fig. 3a). The yellow to orange in the overlay image indicates that

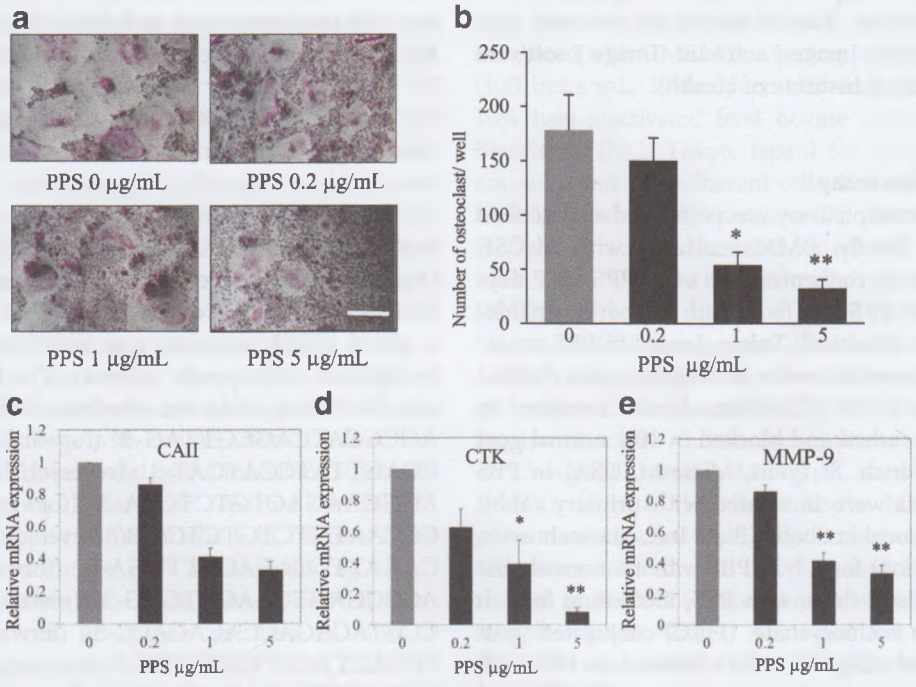

Fig. 1 Shows inhibitory effect of NaPPS on canine OC differentiation. **a** The cells were treated with various concentration of NaPPS followed by M-CSF (20 ng/mL) and RANKL (50 ng/mL) for 7 days. The cells were stained for TRAP stain and **b** TRAP-positive cells (≥3 nuclei) were counted. Scale bar- 100 μm. Bar graphs show the concentration effects of NaPPS on mRNA expression levels of **c** CA II, **d** CTK and **e** MMP-9 determined by real-time PCR and results were normalized to the expression of GAPDH. Data are representative of five independent experiments and expressed as means ± SE. Means with *are significantly different from 0 μg/mL of NaPPS (*$p < 0.05$, **$p < 0.01$)

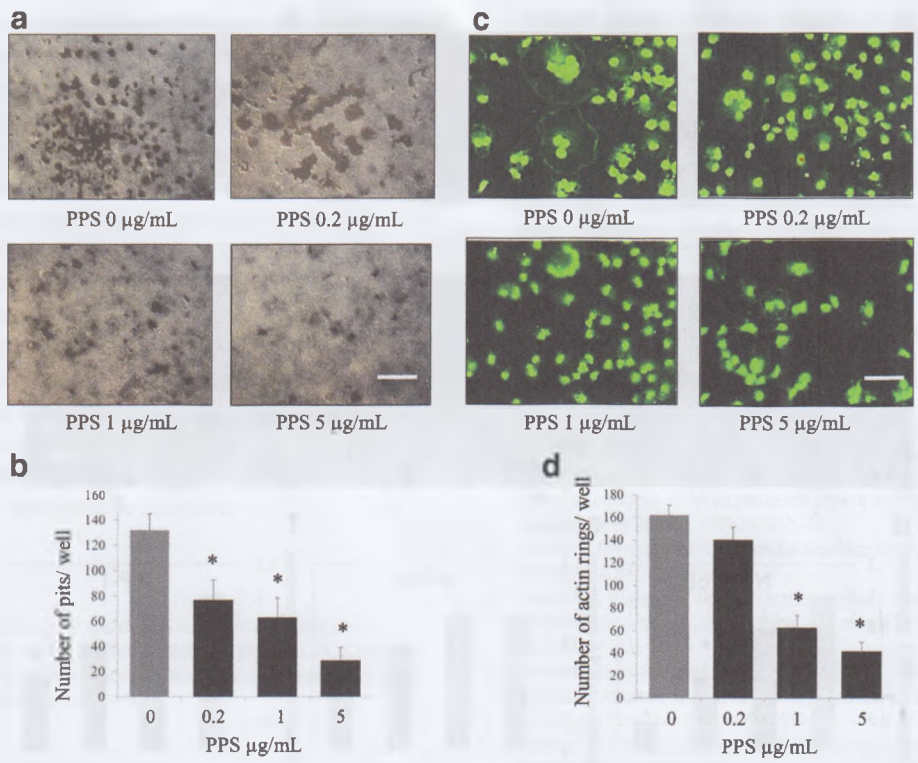

Fig. 2 NaPPS inhibits bone resorption and actin ring formation by concentration gradient. Canine BMMs, cultured with M-CSF (25 ng/mL) and RANKL (50 ng/mL) for 7 days with or without indicated doses of NaPPS. **a** The cells were washed and the resorption pits were counted. **b** The numbers of pits were analyzed with Image-J software. **c** Cells were fixed, stained for F-actin formation (top) and **d** osteoclasts with actin ring were counted (bottom). Scale bar- 200 μm. Column indicates means ± SE of three experiments performed in triplicate. Means with *are significantly different from 0 μg/mL of NaPPS (*$p < 0.05$)

NaPPS and c-Jun proteins are in the same location (Fig. 3b). Pentosan inhibits the expression of NFATc1 at 1 and 5 μg/mL ($p < 0.05$) (Fig. 3d, e, f). And decrease c-Fos and AP-1 activation was identified only at concentration of 5 μg/mL ($p < 0.05$). As shown in Fig. 3c, semi quantitative RT-PCR was correlated with quantitative PCR.

Discussion

In the present study, NaPPS at concentration of 5 μg/mL exerted an inhibitory effect on canine osteoclastogenesis through suppression of key transcription factors such as NFATc1, c-Fos while visualizing co-localization patterns. This information may partially support the suggestion that NaPPS may exert its inhibitory effect on OC by direct interaction with transcription factors, subsequently deterring the target genes like CTK and MMP-9 which are needed in bone resorption activity of OC. To further study the effects of NaPPS on osteoclastogenesis, we examined whether NaPPS affected RANKL-induced OC function by bone resorption assays and actin formation. The results suggested that NaPPS at concentration of 1 and 5 μg/mL suppressed RANKL-induced bone resorption activity and formation of actin-rings of matured OC. The stimulation of M-CSF and RANKL make mature OC result in resorption lacunae, pit formation and actin ring formation [30] which is a prerequisite for OC bone resorption and is the most obvious character of mature OC during osteoclastogenesis [31]. The outcome of this study suggests that the inhibitory action of NaPPS over OC differentiation and function could be applied in treatment of pathological bone disorders where OC play central role.

In this study, the noted inhibition of OC formation, TRAP activity and density of pits at 1 and 5 μg/mL of NaPPS indicated that an inhibitory effect on osteoclastogenesis and function of mature OC. The commonly used phenotype marker, TRAP is expressed particularly in OC and positive for TRAP stain after pre-OC cells differentiation with the supplement of RANKL [27]. Detection of TRAP-positive cell formation is a renowned method of determining OC formation and function [32, 33].

In our study, NaPPS at higher concentrations significantly suppressed the NFATc1 up-regulation in OC normally seen with RANKL treatment. In previous knock-out experiments have been demonstrated that NFATc1 [34, 35] and c-Fos are important transcription factors for RANKL-mediated OC differentiation, fusion, and activation [33]. In addition, previous reports demonstrated

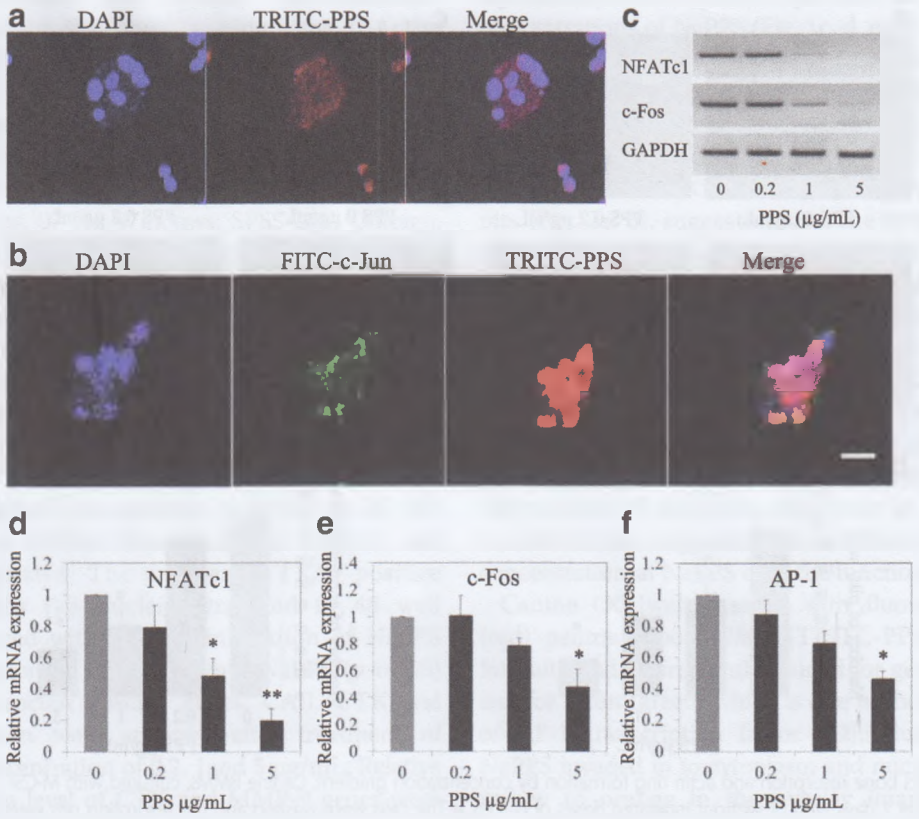

Fig. 3 Inhibitory and co-localization patterns of NaPPS with actively transcribed genes. Osteoclast differentiated from canine bone marrow with supplement of M-CSF (25 ng/mL) RANKL (50 ng/mL) together with absence and presence of NaPPS (0.2, 1, 5 μg/mL) for 7 days was done. **a** Confocal microscopic images of invasion of TRITC-labeled NaPPS in to osteoclast and **b** localization with c-Jun (subunit of AP-1 transcription complex) are shown. The orange in the overlay image indicates that NaPPS and gene activation proteins c-Jun (green) are in the same location. Nuclei were stained with 4,6-diamidino-2-phenylindole dye (DAPI). Scale bar- 100 μm. **c** Attenuation effect of NaPPS on master regulators of osteoclastogenesis (NFATc1, c-Fos) by dose dependent pattern are shown. **d, e** and **f** Relative mRNA expression of NFATc1, c-Fos and AP-1 according to the NaPPS concentration gradient was significantly attenuated at 5 μg/mL. Data expressed as mean ± SE for each PPS concentration after normalizing for the expression of the GAPDH. Means with *are significantly different from 0 μg/mL of NaPPS (*$p < 0.05$, **$p < 0.01$)

that NFATc1 is not induced by RANKL stimulation in OC lacking c-Fos [36]. Further, NFATc1 is the master regulator of osteoclastogenesis which is regulated by the AP-1 complex [37]. Dimeric transcription factor, AP-1 is composed of members of the Jun and Fos protein family [38] and has a massive impact on OC differentiation and production of soluble mediators in bone erosion [39].

Intracellular colocalization and interaction of NaPPS with c-Jun transcriptional factor were observed in this study by immunofluorescence assay emphasizing that the site of action of drug of interest. Binding of c-Fos to the NFATc1 promoter is important for its activation [40]. Suppression of NFATc1 by NaPPS is the consequence of the down-regulation of c-Fos, with the subsequent down-regulation of AP-1 activity and attenuation of OC–specific gene expression required for efficient OC differentiation and bone resorption. Further extension of the study up to detailed work by evaluating specific binding affinity of NaPPS with specific protein at nuclear, sub nuclear domain or nuclear speckles in OC would be much awarded the NaPPS as therapeutic perspective.

Outcome of the present study confirmed that the inhibitory effect of higher concentrations of NaPPS on canine OC differentiation and function, while additional investigations would be required to clarify the mechanism of action of NaPPS on OC in more detail. Although the impact of NaPPS on cell signaling pathways of chondrocytes were well-recognized, invasion in to the OC and its intracellular reactions, competence of osteoclastogenesis from stem cells and effect on functioning structural formation (actin ring) and transcriptional factors have not been considered until probing by this study. To our knowledge, this is the first study to demonstrate that the inhibitory effect of NaPPS on canine bone marrow-derived OC differentiation and bone resorption.

Conclusions

In this study, we examined the inhibitory effect of NaPPS on in vitro cultured canine OC differentiation and function stimulated by RANKL and M-CSF. Our findings provide useful preliminary information on the concentration of this drug and should help increase understanding and awareness of the opportunity and or limitation of its therapeutic use among dogs. In particular, the inhibitory effects of NaPPS on CTK, MMP-9, NFATc1, c-Fos and AP-1in OC could translate to it beneficial effects in the prevention of osteoporosis and other bone-erosive diseases such as rheumatoid arthritis and bone diseases associated with excessive bone resorption. Furthermore, our results would be a flat form and promising launch for further investigation to identify the intracellular acting sites of NaPPS and more detailed protein interactions for detailed therapeutic mechanism of action.

Abbreviations

DMOADs: Disease -modifying osteoarthritis drugs; M-CSF: Macrophage colony-stimulating factor; NaPPS: Sodium pentosan polysulfate; OC: Osteoclast; RA: Rheumatoid arthritis; RANKL: Receptor activator of NF kappa B ligand; TRAP: Tartrate-resistant acid phosphatase

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Authors' contributions

SW was responsible for the study concept, design, conducting all experiments, acquisition of data and all the data analysis and drafting of the manuscript. ECB, JF collected, analyzed and interpreted the data. SK, KH was involved in design of study concept, drafting and revising of the manuscript. MO was a major contributor in conception and design of the study, interpretation of data, drafting and revising the manuscript. All authors contributed to the interpretation of the data and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Aspirin eugenol ester regulates cecal contents metabolomic profile and microbiota in an animal model of hyperlipidemia

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Abstract

Background: Hyperlipidemia, with an increasing of prevalence, has become one of the common metabolic diseases in companion animal clinic. Aspirin eugenol ester (AEE) is a novel compound that exhibits efficacious anti-hyperlipidemia activities. However, its mechanisms are still not completely known. The objective of present study was to investigate the intervention effects of AEE on cecal contents metabonomics profile and microbiota in hyperlipidemia rats.

Results: Three groups of rats were fed with a control diet, or high fat diet (HFD) containing or not AEE. The results showed the beneficial effects of AEE in HFD-fed rats such as the reducing of aspartate aminotransferase (AST) and total cholesterol (TCH). Distinct changes in metabonomics profile of cecal contents were observed among control, model and AEE groups. HFD-induced alterations of eight metabolites in cecal contents mainly related with purine metabolism, linoleic acid metabolism, glycerophospholipid metabolism, sphingolipid metabolism and pyrimidine metabolism were reversed by AEE treatment. Principal coordinate analysis (PCoA) and cluster analysis of microbiota showed altered patterns with distinct differences in AEE group versus model group, indicating that AEE treatment improved the negative effects caused by HFD on cecal microbiota. In addition, the correction analysis revealed the possible link between the identified metabolites and cecal microbiota.

Conclusions: This study showed regulation effects of AEE on cecal contents metabonomics profile and microbiota, which could provide information to reveal the possible underlying mechanism of AEE on hyperlipidemia treatment.

Keywords: Aspirin eugenol ester, Gut microbiota, Metabonomics, Cecal contents, Hyperlipidemia, UPLC-Q-TOF/MS, High fat diet

Background

As an emerging discipline, metabonomics provide a powerful approach to discover biomarkers in biological systems [1]. Based on the measurement of global metabolite profiles, metabonomics has been increasingly applied to investigate the responses of living systems to genetic modifications or pathophysiological stimuli. At present, liquid chromatography-mass spectrometry (LC-MS) has become one of the frequently used techniques in metabonomics studies for its numerous advantages such as high sensitivity and reproducibility [2]. Gut microbiota is now considered as a vital factor for human health and disease [3]. It has been recognized that gut microbiota plays important roles in many key functions of the host, which are associated with reproduction, obesity, cancer, nutrition restriction and gut immune maturation [4, 5].

Hyperlipidemia has now become a serious health issue in human and companion pets such as dogs and cats [6,

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7]. In animals, as a health risk factor, hyperlipidemia is involved in the progress of many diseases such as inflammation, diabetes mellitus, obesity, atherosclerosis and hypertension. Many studies suggest that the disorder of lipid metabolism is one of the main features of hyperlipidemia, which can lead the abnormal levels of triglycerides (TG), total cholesterol (TCH), low-density lipoprotein (LDL) and high-density lipoprotein (HDL). In the market, many drugs such as statins, nicotinic acid and fibrates are commonly used for hyperlipidemia treatment. But unfortunately, some side or toxic effects of these drugs, for instance, statin-induced myopathy and fibrate-induced rhabdomyolysis, have limited their clinical application [8]. Therefore, to develop a safe and effective drug for hyperlipidemia treatment is becoming a research hotspot in the world.

As we all known, aspirin is widely used for the treatment of inflammation, fever, arthritis and the prevention of cardiovascular disease. Moreover, some studies indicate that aspirin has therapeutic effects on dyslipidemia and related diseases [9]. A number of reports have demonstrated that eugenol has remarkable anti-hyperlipidemia effect such as the improvement of abnormal lipid profiles in rats fed with high fat diet (HFD) [10, 11]. However, the side effects such as gastrointestinal damage of aspirin and vulnerability of eugenol limit their application. These disadvantages are mainly caused by the carboxyl group of aspirin and hydroxyl group of eugenol. In order to reduce side effect and improve stabilization through chemical structural modification, aspirin eugenol ester (AEE), a pale yellow and odourless crystal, was synthesized with the starting precursors of aspirin and eugenol according to the pro-drug principle [12]. Many studies including toxicity, teratogenicity, metabolism, pharmacodynamics and stability of AEE have been carried out in our lab, and the results indicate that AEE is a promising compound with good druggability [13–15].

We previously established the hyperlipidemia model in rats induced by HFD, and investigated the regulation effects of AEE on blood lipids [16, 17]. Moreover, the effects of AEE on the metabonomics profiles of plasma, urine, liver and feces were also explored [18, 19]. The results indicated that AEE was an effective compound for hyperlipidemia treatment, and its mechanism could be partly revealed by the metabonomics study. As important biological samples, the relative abundances of many metabolites in cecal contents are different from those in the feces, which are attractive for biomarker investigation to illustrate the therapeutic basis of drug [20, 21]. It is well known that the concentration and diversity of the microbial communities depend on the sample used [22]. Some studies showed that the bacterial diversity, richness and community composition of fecal samples were low compared to the cecal contents [23]. Little information is known concerning the alteration of cecal contents metabonomics and microbiota associated with AEE therapeutic effects. With the application of ultra performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF/MS) analysis and 16S rRNA Illumina sequencing, the objective of this follow-up study was to investigate the effects of AEE on cecal contents metabonomics profile and microbiota and find out more evidences to understand the possible underlying mechanism of AEE against hyperlipidemia.

Results

Body growth, liver weight and food consumption

There were no differences in the initial or final body weights or liver/body weight ratio among control, model and AEE groups (Fig. 1a and b). Notably, the mean value of liver/body weight ratio in the model group was higher than those in the control and AEE group, but not significant (Fig. 1b). Daily food consumption in the model and AEE groups were significantly decreased ($P < 0.05$) compared to the control (Fig. 1c). There was no statistical difference in food consumption between model group and AEE group ($P > 0.05$).

Hematological and serum biochemical parameters

Effect of AEE on hematological parameters was shown in Table 1. No significant differences were observed in hematological parameters except platelet (PLT) index. PLT was significantly higher in model group in comparison with the control group ($P < 0.01$), as well as in AEE group ($P < 0.05$). Compared with the model group, AEE treatment reduced the increase of PLT, but there was no significance in statistics.

When compared with the control rats, the results of biochemical parameters indicated that HFD showed strong effects on increasing alanine aminotransferase (ALT), alkaline phosphatase (ALP), TCH, TG and LDL levels, and reducing direct bilirubin (DB) and urea (Table 2, $P < 0.01$). ALP and ALT levels were significantly higher in the AEE group than those in the control group ($P < 0.01$), whereas the creatine kinase (CK), lactate dehydrogenase (LDH), DB, urea and HDL was significantly reduced ($P < 0.05$ and $P < 0.01$). In comparison with the model group, aspartate aminotransferase (AST), LDH and TCH levels in AEE group were significantly reduced ($P < 0.05$ and $P < 0.01$), indicating the partial improvement of biochemical profile in HFD-fed rats. Atherosclerosis index (AI) values in model and AEE groups were significantly increased in comparison with the control ($P < 0.01$, Fig. 1d). No statistical difference of AI was observed between model and AEE groups.

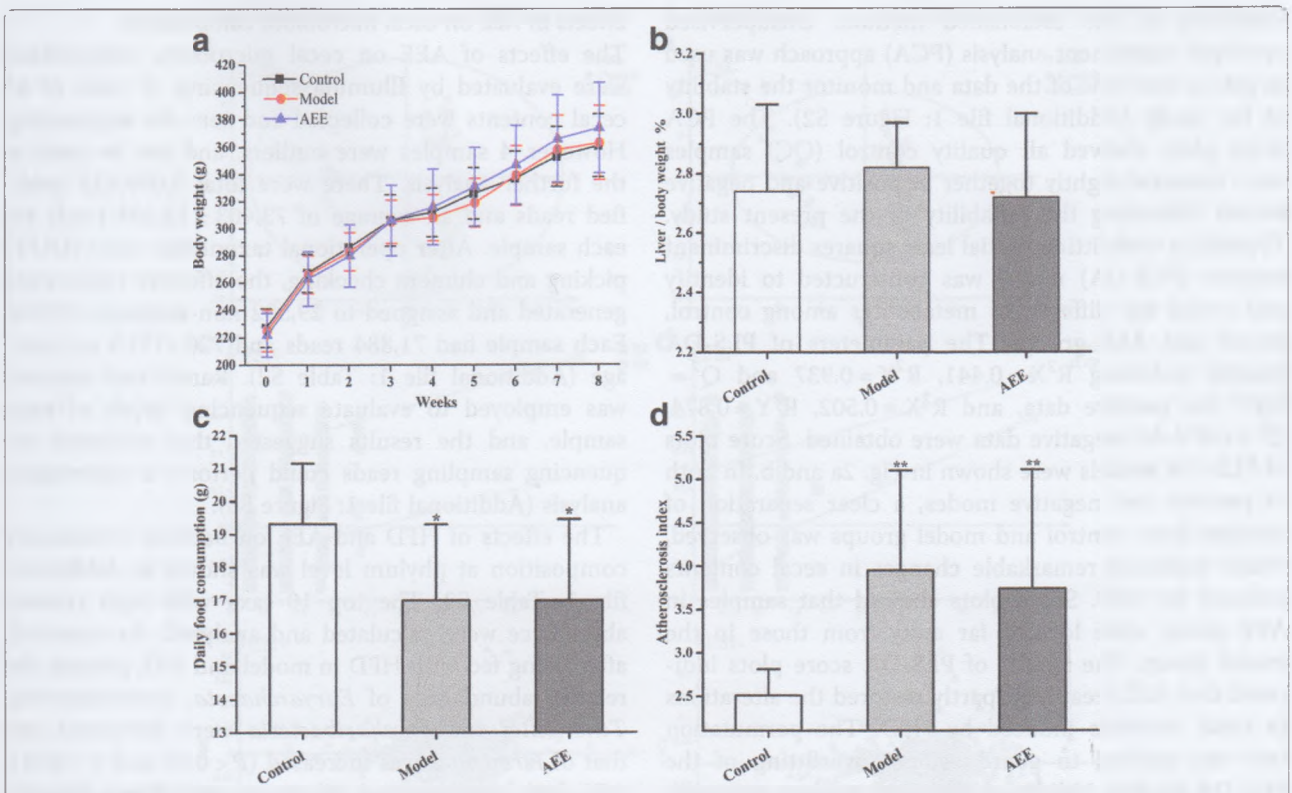

Fig. 1 Effects of AEE on hyperlipidemia related indices in rats ($n = 10$). **a** Effects of AEE on body weight. **b** Effects of AEE on liver weight. **c** Effects of AEE on daily food consumption. **d** Effects of AEE on atherosclerosis index. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group

Cecal contents metabolic profiling

In this study, an UPLC-Q/TOF MS-based cecal contents metabonomics study was carried out in rats fed with HFD. Representative total ion chromatograms (TICs) of the cecal contents in positive and negative modes were shown in Additional file 1: Figure S1, which displayed good separation effect and strong

Table 1 Hematological findings in rats fed with HFD supplemented with AEE

Variables	Units	Control	Model	AEE
WBC	10^9/L	82.1 ± 8.4	87.7 ± 9.4	84.6 ± 6.9
LY	10^9/L	6.77 ± 1.27	7.74 ± 1.82	7.08 ± 1.10
MONO	10^9/L	1.84 ± 0.30	2.05 ± 0.35	1.93 ± 0.22
NEUT	10^9/L	73.7 ± 7.0	77.9 ± 7.5	75.6 ± 5.7
RBC	10^{12}/L	9.24 ± 0.96	8.91 ± 0.58	9.16 ± 0.92
PLT	10^9/L	1034 ± 172	1271 ± 220**	1107 ± 143*
HCT	%	51.4 ± 5.4	48.9 ± 4.2	50.5 ± 5.0
MCV	fL	55.8 ± 2.6	54.9 ± 2.1	55.2 ± 2.5
RDW-CV	%	14.3 ± 0.6	14.8 ± 0.7	14.6 ± 0.8
MPV	fL	5.73 ± 0.37	5.76 ± 0.42	5.52 ± 0.28
PDW	%	16.3 ± 0.2	16.4 ± 0.3	16.2 ± 0.2

Values were expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group

Table 2 Serum levels of biochemical parameters of rats in different group

Variables	Units	Control	Model	AEE
TB	μmol /L	1.45 ± 0.28	1.46 ± 0.24	1.38 ± 0.23
DB	μmol /L	1.32 ± 0.47	0.96 ± 0.23**	0.93 ± 0.21**
TP	g/L	56.8 ± 4.5	56.9 ± 3.5	57.2 ± 3.7
ALB	g/L	35.2 ± 3.0	34.3 ± 1.7	34.7 ± 2.0
GLOB	g/L	21.7 ± 1.8	22.6 ± 2.3	22.5 ± 2.1
ALT	U/L	36.1 ± 3.5	48.2 ± 8.5**	50.3 ± 7.3**
AST	U/L	125.3 ± 18.1	134.6 ± 29.3	116.7 ± 16.1#
ALP	U/L	108.3 ± 19.4	152.2 ± 24.2**	156.2 ± 27.3**
LDH	U/L	1037 ± 175	1145 ± 267	853 ± 152*#
CK	U/L	819 ± 163	913 ± 328	694 ± 148*
Urea	mmol/L	7.66 ± 0.68	5.58 ± 0.60**	5.68 ± 0.87**
CREA	μmol /L	41.8 ± 8.5	40.2 ± 5.4	41.5 ± 4.1
GLU	mmol/L	6.56 ± 1.26	6.94 ± 1.32	7.35 ± 1.21
TG	mmol/L	1.10 ± 0.30	1.41 ± 0.19**	1.27 ± 0.22
TCH	mmol/L	1.20 ± 0.08	1.44 ± 0.17**	1.29 ± 0.08##
HDL	mmol/L	0.46 ± 0.05	0.42 ± 0.05	0.42 ± 0.12
LDL	mmol/L	0.37 ± 0.05	0.44 ± 0.05**	0.40 ± 0.03

Values were expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the model group

sensitivity of the established method. Unsupervised principal component analysis (PCA) approach was used to get an overview of the data and monitor the stability of the study (Additional file 1: Figure S2). The PCA score plots showed all quality control (QC) samples were clustered tightly together in positive and negative modes indicating the reliability of the present study. Typically,a well-fitting partial least squares discriminant analysis (PLS-DA) model was constructed to identify and reveal the differential metabolites among control, model and AEE groups. The parameters of PLS-DA models including $R^2X = 0.441$, $R^2Y = 0.937$ and $Q^2 = 0.497$ for positive data, and $R^2X = 0.502$, $R^2Y = 0.874$, $Q^2 = 0.674$ for negative data were obtained. Score plots of PLS-DA models were shown in Fig. 2a and b. In both of positive and negative modes, a clear separation of samples from control and model groups was observed, which indicated remarkable changes in cecal contents induced by HFD. Score plots showed that samples in AEE group were located far away from those in the model group. The results of PLS-DA score plots indicated that AEE treatment partly restored the alterations in cecal contents induced by HFD. The permutation test was applied to guard against overfitting of the PLS-DA models. Validation with 200 random permutation tests generated intercepts of $R^2 = 0.375$ and $Q^2 = -0.211$ from positive model data (Fig. 2c) and $R^2 = 0.272$ and $Q^2 = -0.279$ from negative model data (Fig. 2d), which demonstrated that the PLS-DA models were robust without overfitting.

Differential metabolites in cecal contents

In PLS-DA models, loading-plot as a tool was used to identify the metabolites contributing to group separation. As shown in Fig. 2e and f, ions in loading-plot away from center were considered as potential biomarkers responsible group separation. With variance importance for projection (VIP) values above 1 and adjusted *P*-values less than 0.05, 8 metabolites were filtered and identified as potential biomarkers (Table 3). Compared with the control rats, HFD significantly increased the relative intensities on some potential biomarkers including lysophosphatidylcholine (LysoPC) (18:1(9Z)), linoleic acid, linoleoyl ethanolamide, oleamide and sphingosine, and the biomarkers like hypoxanthine, uridine and sebacic acid were significantly reduced ($P < 0.05$ and $P < 0.01$). Notably, AEE treatment partly reversed the abnormal metabolite changes in cecal contents induced by HFD such as the significant reduction of LysoPC (18:1(9Z)) and sphingosine. The pathway results from KEGG revealed that the disturbed pathways in cecal contents were purine metabolism, linoleic acid metabolism, glycerophospholipid metabolism, sphingolipid metabolism and pyrimidine metabolism.

Effects of AEE on cecal microbiota composition

The effects of AEE on cecal microbiota composition were evaluated by Illumina sequencing. A total of 45 cecal contents were collected and sent for sequencing. However, 4 samples were outliers, and not be used in the further analysis. There were total 3,009,532 qualified reads and an average of 73,403 ± 13,104 reads for each sample. After operational taxonomic units (OUT) picking and chimera checking, the effective reads were generated and assigned to 29,532 non-singleton OTUs. Each sample had 71,884 reads and 720 OTUs on average (Additional file 1: Table S2). Rarefaction analysis was employed to evaluate sequencing depth of each sample, and the results suggested that sufficient sequencing sampling reads could perform a meaningful analysis (Additional file 1: Figure S3).

The effects of HFD and AEE on bacteria community composition at phylum level was shown in Additional file 1: Table S3. The top 10 taxa with high relative abundance were calculated and analyzed. As expected, after being fed with HFD in model and AEE groups, the relative abundances of *Euryarchaeota*, *Actinobacteria*, *Tenericutes* and *Saccharibacteria* were decreased and that of *Firmicutes* was increased ($P < 0.05$ and $P < 0.01$). AEE had some reversal effects on microbiota disturbance induced by HFD such as the reduction of *Firmicutes* and the increase of *Euryarchaeota*, but no statistical difference in taxa abundances was observed between AEE and model groups.

At the genus level, ten key microbial genera associated with AEE treatment in the rats fed with HFD were found (Table 4). These genera were selected based on relative abundance and statistical difference, which were responsible for the difference among three groups. Interestingly, significant differences in the cecal contents microbiota of rats fed with HFD were observed. For example, when compared with the control rats, *Corynebacterium_1*, *Nosocomiicoccus*, and *Jeotgalicoccus* were significantly decreased in the rats fed with HFD ($P < 0.01$). A similar decrease of these generas was also observed in AEE group in comparison with the model group ($P < 0.01$). Notably, rats fed with HFD had higher levels of *Turicibacter* and *Bifidobacterium* compared with the control ($P < 0.01$). Remarkable increase of *Bifidobacterium* and reduction of *Turicibacter* were found in AEE group than those in the model ($P < 0.05$). In addition, there was no difference in the relative abundance of *Staphylococcus*, *[Eubacterium]_brachy_group*, *[Ruminococcus]_gauvreauii_group*, *Ruminococcaceae_NK4A214_group* and *[Eubacterium]_xylanophilum_group* between control and model groups. However, AEE had significant influence on these generas such as the increase of *[Ruminococcus]_gauvreauii_group* and the reduction of *Staphylococcus*.

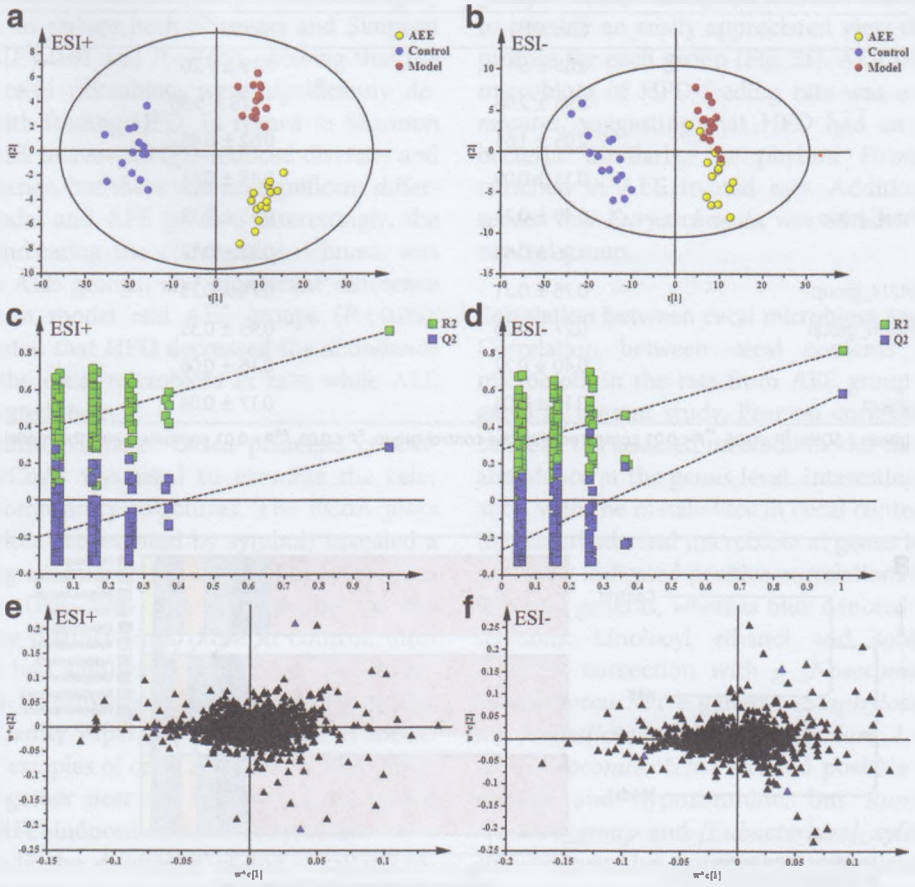

Fig. 2 Multivariate data analyses of cecal contents based on UPLC-Q-TOF/MS analysis. ESI+: electrospray ionization in positive ion mode; ESI-: electrospray ionization in negative ion mode. **a** and **b** PLS-DA score plots in positive and negative modes. ESI+: $R^2X = 0.441$, $R^2Y = 0.937$, $Q^2 = 0.497$; ESI-: $R^2X = 0.502$, $R^2Y = 0.874$, $Q^2 = 0.674$. **c** and **d** Plots of the permutation test of the PLS-DA models. ESI+: $R^2 = 0.375$, $Q^2 = -0.211$; ESI-: $R^2 = 0.272$, $Q^2 = -0.279$. **e** and **f** Loading plots of the PLS-DA models

AEE altered cecal microbiota structure

A phylogenic tree analysis based on the unweighted pair-group method with arithmetic mean (UPGMA) was used to cluster the cecal samples in different group. Figure 3a showed that samples in control and AEE groups were grouped closely, and samples in model group were branched separately. These results suggested that the microbial communities in AEE groups were more similar to the control than model. Next, changes in microbial communities were investigated using alpha diversity measures including Shannon's diversity index and Simpson (estimated OTUs) (Fig. 3b and c). Significant differences were

Table 3 Potential biomarkers in cecal contents based on the UPLC-Q-TOF/MS analysis and the changes between different groups

SM	RT	VIP	Metabolite	Formula	m/z	Adduction	Fold change		Pathway
							M/C	AEE/M	
+	2.26	2.92	Hypoxanthine	$C_5H_4N_4O$	137.0459	$[M+H]^+$	0.39**	1.12	Purine metabolism
+	18.11	2.04	Linoleic acid	$C_{18}H_{32}O_2$	281.2479	[M + H]+	3.78**	0.81	Linoleic acid metabolism
+	17.84	1.40	LysoPC(18:1(9Z))	$C_{26}H_{52}NO_7P$	522.3606	[M + H]+	1.90*	0.47*	Glycerophospholipid metabolism
+	19.32	1.01	Linoleoyl ethanolamide	$C_{20}H_{37}NO_2$	324.2905	[M + H]+	1.59*	0.88	–
+	21.26	2.51	Oleamide	$C_{18}H_{35}NO$	282.2796	[M + H]+	2.19*	0.49*	–
+	14.81	8.12	Sphingosine	$C_{18}H_{37}NO_2$	300.2904	[M + H]+	2.98**	0.52**	Sphingolipid metabolism
–	2.99	1.07	Uridine	$C_9H_{12}N_2O_6$	243.0622	[M-H]-	0.35**	1.14	Pyrimidine metabolism
–	10.40	1.17	Sebacic acid	$C_{10}H_{18}O_4$	201.1132	[M-H]-	0.49*	1.43	–

SM scan model, *RT* retention time, *VIP* Variance importance for projection, *LysoPC*, Lysophosphatidylcholine, *M/C*: model versus control, AEE/M: AEE versus model, $^*P < 0.05$, $^{**}P < 0.01$ compared with corresponding group

Table 4 The relative abundance of key different genera in rats

Genus	Control	Model	AEE
Staphylococcus	8.05 ± 5.17	8.19 ± 7.26	2.94 ± 1.45$^{**\#\#}$
Turicibacter	8.95 ± 2.98	13.78 ± 5.36**	9.46 ± 3.03$^{\#}$
Corynebacterium_1	2.05 ± 1.63	0.62 ± 0.48**	0.23 ± 0.16$^{**\#\#}$
Bifidobacterium	0.13 ± 0.09	0.43 ± 0.31**	1.29 ± 1.02$^{**\#}$
[Ruminococcus]_gauvreauii_group	0.40 ± 0.21	0.43 ± 0.15	0.95 ± 0.56$^{\#\#}$
Nosocomiicoccus	1.26 ± 0.48	0.42 ± 0.12**	0.18 ± 0.13$^{**\#\#}$
Ruminococcaceae_NK4A214_group	0.76 ± 0.31	0.79 ± 0.23	1.27 ± 0.38$^{\#\#}$
[Eubacterium]_xylanophilum_group	0.77 ± 0.26	0.93 ± 0.35	1.27 ± 0.24$^{\#\#}$
Jeotgalicoccus	0.40 ± 0.22	0.16 ± 0.06**	0.06 ± 0.05$^{**\#\#}$
[Eubacterium]_brachy_group	0.16 ± 0.03	0.17 ± 0.04	0.13 ± 0.03$^{\#\#}$

Data were expressed as (mean ± SD)%. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the model group

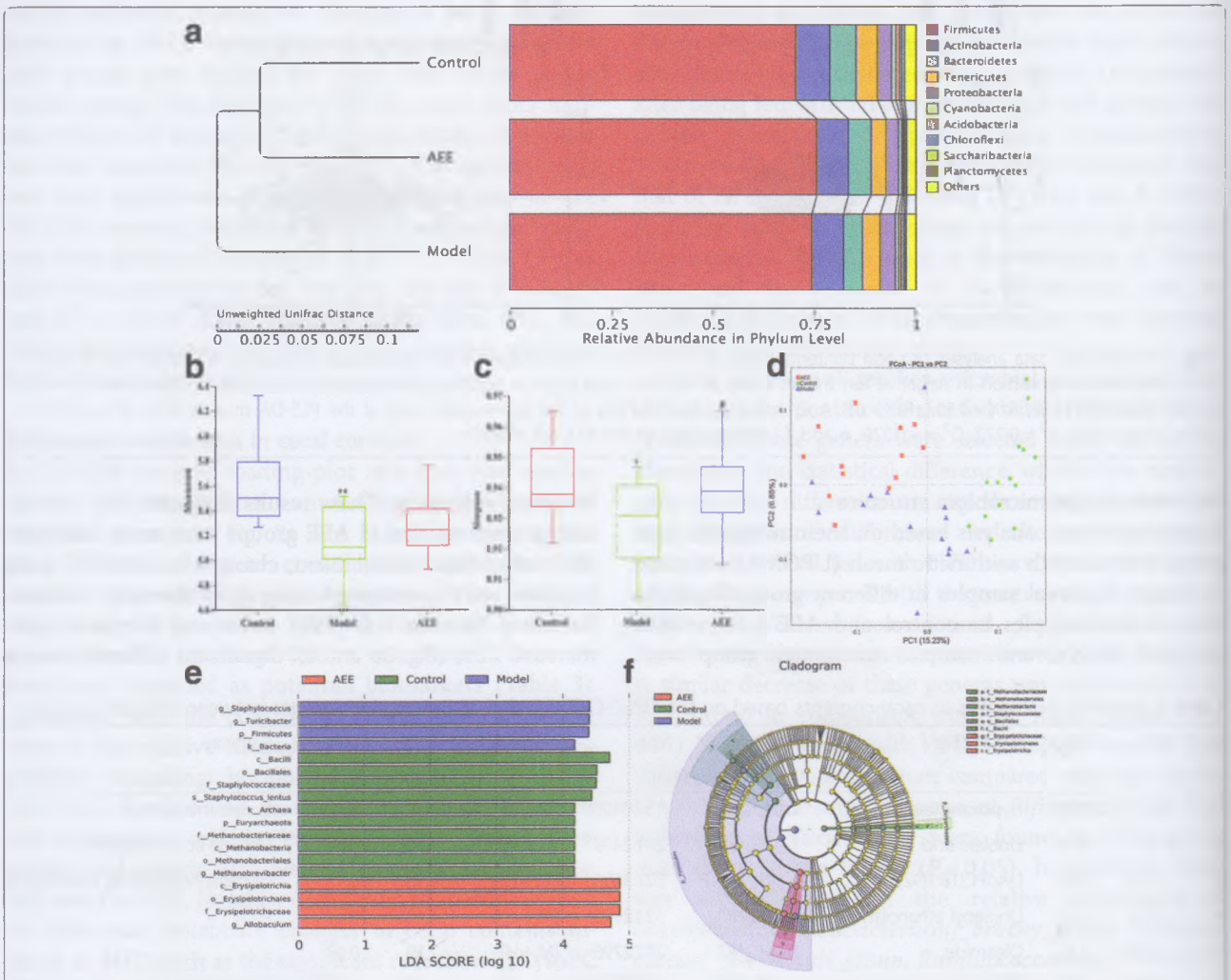

Fig. 3 AEE reversed the dysbiosis of caecal microbiota in HFD-induced hyperlipidemia rats. **a** Cluster analysis of the samples based on UPGMA. **b** and **c** Community diversity of each group reflected by Shannon and Simpson indexes with Wilcoxon analysis. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control group; $^{\#}P < 0.05$ compared with the model group. **d** Principal coordinate analysis (PCoA) of bacterial community structures of the gut microbiota of each group. **e** LDA scores as calculated by LEfSe analysis. Only taxa with LDA scores of more than 4 were presented. **f** LEfSe cladogram representing different abundant taxa

found between control and model groups. HFD had highly significant effects to reduce both Shannon and Simpson diversity indexes ($P < 0.01$ and $P < 0.05$), showing that the diversity of the cecal microbiota were significantly decreased in rats with feeding HFD. In regard to Shannon diversity index, AEE increased HFD-reduced diversity and reduced the difference, but there was no significant difference between model and AEE groups. Interestingly, the Simpson index, indicating the community richness, was also increased in AEE groups, and significant difference was found between model and AEE groups ($P < 0.05$). These data suggested that HFD decreased the abundance and diversity of the cecal microbiota in rats, while AEE treatment ameliorated them.

Unweighted unifrac distance based principal coordinates analysis (PCoA) was used to examine the relationship of the community structures. The PCoA plots (sample's microbiota represented by symbol) revealed a distinct clustering of microbiota composition for each treatment group (Fig. 3d). The microbiotas of the model group were distinct from those in control, indicating that HFD had significant impact on the microbiota community. Meanwhile, samples in AEE groups were also significantly separated from those in model group. Notably, 7 samples of cecal contents in AEE group were clustered together near the control suggesting the improvement of HFD-induced microbiota dysbiosis.

In order to study the difference of the cecal microbiota, a linear discriminant analysis effect size (LEfSe) with LDA score at least 4 was performed. *Archaea, Euryarchaeota* and *Methanobrevibacter* were found more in the control group (Fig. 3e). Greater proportions of *Turicibacter and Staphylococcus* were enriched in the model than in the control (Fig. 3e). The taxonomic abundances in the cecal microbiota of the model and AEE groups were also compared with LEfSe analysis. Figure 3e showed that bacteria taxa such as *Allobaculum* and *Erysipelotrichaceae* were different between model and AEE groups. LEfSe cladogram of bacterial lineages was used to provide an easily appreciated view of the enrichment profiles for each group (Fig. 3f). At a phylum level, cecal microbiota of HFD-feeding rats was enriched with *Firmicutes*, suggesting that HFD had an impact on these bacteria. Similarly, the phylum *Firmicutes* were also enriched in AEE treated rats. Additionally, it was observed that *Euryarchaeota* was enriched in rats from the control group.

Correlation between cecal microbiota and metabolites

Correlation between cecal contents metabolites and microbiota in the rats from AEE group was also investigated in present study. Pearson correlation was analyzed between the selected metabolites and the cecal microbiota abundance at the genus level. Interestingly, a clear correlation with the metabolites in cecal contents was found for the disturbed cecal microbiota at genus level. In Fig. 4, the red color indicated positive correlations between metabolites and generas, whereas blue denoted the negative correlations. Linoleoyl ethanol and sphingosine showed negative correction with *g__Nosocomiicoccus* and *g__Jeotgalicoccus*. Five generas (*Staphylococcus*, *Turicibacter*, *Jeotgalicoccus*, *Corynebacterium_1*, *Bifidobacterium* and *Nosocomiicoccus*) showed positive correlation with uridine and hypoxanthine, but *Ruminococcaceae_NK4A214_group* and *[Eubacterium]_xylanophilum_group* showed negative correlation with uridine and linoleic acid. Sebacic acid was positively correlated with *Staphylococcus* and *Corynebacterium_1*. However, sebacic acid showed no correlation with *Nosocomiicoccus* and *Jeotgalicoccus*, and a similar result was observed among linoleic acid, *Nosocomiicoccus*, *Jeotgalicoccus* and *[Eubacterium]_brachy_group*.

Discussion

As one of the most common metabolic disease, pathophysiology of hyperlipidemia is very complex and has

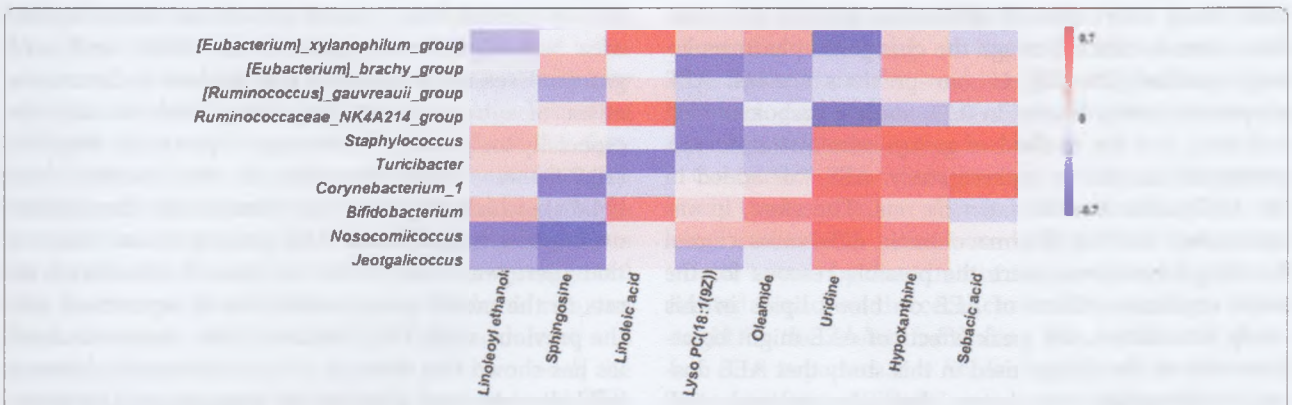

Fig. 4 Pearson correlation between caecal microbiota (at the genera level) and potential biomarker affected by AEE treatment. Red color indicating positive correlations whereas blue denoting negative correlations

been only partially elucidated. Previous study reported that the development of hyperlipidemia was accompanied with the changes of gut microbiota and metabonomics profile [24]. AEE consists of two chemical structural units from aspirin and eugenol. Pharmacokinetics results showed that AEE was directly decomposed into salicylic acid and eugenol after oral administration, which could exhibit their original activities and act synergistically [13]. In this work, metabonomic analysis and cecal microbiota were applied to systemically evaluate the treatment effects of AEE in hyperlipidemia rats. The results showed that HFD consumption induced significant changes in cecal contents metabolic profiles and microbiota, whereas AEE could reverse the HFD-induced these alterations in hyperlipidemia rats. This study indicated that the integration of metabonomics study and gut microbiota is a sensitive and effective approach in drug development. Moreover, the results of this study could provide additional evidence to illustrate the possible action mechanism of AEE against hyperlipidemia.

Both of aspirin and eugenol have good effects on improving hyperlipidemia induced by HFD [25–27]. Therefore, as the combination of aspirin and eugenol, AEE has stronger therapeutic efficiency than its precursors on hyperlipidemia treatment [16]. In our previous study, serum biochemical results had confirmed that AEE could significantly reduce the levels of TG, LDL and TCH in hyperlipidemic rats induced by the HFD [16, 17]. In present study, the consumption of HFD caused the disorder of lipid profiles such as the increase of TG, TCH and LDL. These results indicated that the hyperlipidemia disease model was established successfully. AEE ameliorated the HFD-induced blood lipid disorder such as the significant reduced levels of TCH, and low values of LDL and TG, which were consistent with the previous results [17]. However, it was noted that the regulation effects of AEE on blood lipid profile in present study were weaker than those in our previous results [16–18]. Evidences from numerous studies have shown that the same drug with different administration way can produce various effects through the changes in pharmacokinetic profiles [28, 29]. In our previous studies, AEE suspension was prepared in 0.5% sodium carboxymethyl cellulose, and the method of gavage administration was performed in rats. In present study, AEE was added in the HFD, and then be eaten by rats. Therefore, it was speculated that the pharmacokinetic differences caused by drug delivery way were the possible reasons for the weak regulation effects of AEE on blood lipids in this study. In addition, the weak effects of AEE might be attributable to the dosage used in this study that AEE dosage (43.5 mg/kg) was lower than the optimal AEE dosage (54 mg/kg) for hyperlipidemia treatment found in our previous research [16].

AI is a reliable index to access the lipid contribution to the cardiovascular disease risk. Elevated AI values in the model group were mainly associated with the HFD-induced lipid orders. However, AEE showed no influence on AI index in present study, which was inconsistent with the previous results found in atherosclerotic hamster [30]. There are several possible reasons for these results. First, different administration method used in the experiment might be the main reason for the poor regulation effects on AI index. Second, the differences of HFD composition, animals and the duration of the experiment may be the other reasons for the AI results. Literature indicated that the lipid metabolism and cholesterol transport of rat and hamster are different [31]. There was no difference in daily food consumption between model and AEE groups. Therefore, the improved lipid profile in AEE group was not related to the food rejection. It was also noticed that there was no difference in body weight between control and model groups, which might be related with inadequate HFD consumption [32].

ALT and AST are important enzymes in the liver, which can be served as indicators of liver function. The mean serum levels of ALT and AST showed an increase in model group after HFD feeding for 8 weeks compared with those in the control group. These changes of ALT and AST showed that the rats should had developed liver function damage. After administration of AEE, the serum AST was significantly reduced, indicating that AEE had positive effect on liver function. Some researchers have reported that the liver weight is increased after the rats fed with HFD, which is related with the pathological changes in the liver such as the edema and steatosis [33]. In present study, although the liver weight was increased in the model group, there was no statistical difference among control, model and AEE groups. It is worth noting that increasing studies have showed that lipid disorders could cause decline of renal function caused by oxidative or pathological damages [34, 35]. In present study, CREA showed no difference and urea was significantly reduced in model and AEE groups. Urea is the principal end product in the metabolism of nitrogen-containing compounds in animals, especially for protein. Percentage of protein in the HFD (18.3%) was lower than that in the standard diet (24.4%), which might be the reasons for the reduced urea levels in model and AEE groups. It was observed that there was a significant increase in platelet of the rats in the model group, which was in agreement with the previous study [36]. Evidence from numerous studies has shown that there is a close relationship between lipid disorder and platelet, for instance, HFD-induced dyslipidemia could lead platelet adhesion and aggregation. [37] Administration of AEE decreased the mean

level of platelet, which might attribute to the ameliorative effects on lipid profiles.

Metabonomics is a sensitive and powerful tool to provide quantitative measures of global changes in the metabolic profile. UPLC-Q-TOF/MS analysis method was used in present study, in conjunction with multivariate data analysis, to identify the metabolites significantly affected by AEE treatment in cecal contents. The metabolomic analysis indicated that there was a significant difference in metabolic patterns of the control, model and AEE groups in the score plots. The results of metabonomics study were partly in agreement with the findings in blood biochemistry and cecal microbiota, indicating the improvement of AEE on hyperlipidemia and the interactions among blood lipid, metabonomics and microbiota. Moreover, 8 metabolites (e.g. hypoxanthine, linoleic acid, sphingosine, uridine, sebacic acid) were selected as potential biomarkers which were associated with sphingolipid metabolism, purine metabolism, linoleic acid metabolism, glycerophospholipid metabolism and pyrimidine metabolism.

Sphingosine is a primary part of sphingolipids, and can be phosphorylated to the formation of sphingosine-1-phosphate. Several publications have demonstrated that increased dietary saturated fat content can elevate sphingolipid metabolism, and the abnormal sphingolipid metabolism is closely associated with obesity and hyperlipidemia [38]. In addition, the inhibitation of sphingolipid metabolism could improve circulating lipids through the reduction of LDL [39]. In our study, sphingosine was increased in hyperlipidemic rats, which was matched with other reports that sphingosine was enhanced in the hamster fed with HFD [40]. Notably, AEE treatment showed favorable inhibition on sphingosine, suggesting that the depression of AEE on sphingolipid metabolism might contribute to its efficacy on hyperlipidemia.

The disturbance of glycerophospholipid and fatty acid metabolism is found to be directly associated with the initiation and progression of hyperlipidemia. In this study, the alterations of potential biomarkers including LysoPC (18:1(9Z)), linoleic acid and oleamide had influence on the metabolism of glycerophospholipid and fatty acid. Linoleic acid, a carboxylic acid, is a polyunsaturated omega-6 fatty acid. Oleamide is an amide of the fatty acid oleic acid and the substrates of fatty acids amide hydrolase. In present study, linoleic acid and oleamide in cecal contents were increased in the hyperlipidemic rats compared with the control rats. These results suggested that the fatty acids oxidation of hyperlipidemic rats was blocked, which could accumulate fatty acid level and cause dyslipidemia [41]. In contrast to the model, the levels of oleamide and linoleic acid were reduced after AEE treatment, which implied that AEE could improve lipid disorders by regulating fatty acid metabolism. LysoPCs, served as precise marker for specific metabolic disease, play important roles in the development of cardiovascular disease by triggering inflammation and the autoimmune response [42]. LysoPC (18:1(9Z)) was increased in the model group, suggesting the glycerophospholipid metabolism was promoted under hyperlipidemia condition. This increase could be significantly inhibited by AEE treatment, implying that the therapeutic effect of AEE on hyperlipidemia might ascribe to the inhibition of glycerophospholipid metabolism. Sebacic acid is a dicarboxylic acid with 10 carbon atoms, which can produce important intermediates of energy metabolism such as acetyl-CoA and succinyl-CoA. Some researchers have reported that sebacic acid was increased in the feces in atherosclerotic rats [43]. Inconsistent with the abovementioned studies, the content of sebacic acid was reduced in the model group, indicating that the HFD might destroy the equilibrium of energy metabolism. AEE treatment could inhibit the down-regulation of sebacic acid, suggesting AEE could ameliorate the disturbed energy metabolism.

Metabolites such as uridine and hypoxanthine related to purine and pyrimidine metabolism were also identified in the study. Uridine and hypoxanthine are pyrimidine and purine derivatives, respectively. Several recent publications have demonstrated that hypoxanthine and uridine could be significantly reduced in the liver of the obese mice under HFD [44], and the hypoxanthine in the feces was also decreased by the microfloral population reduction [45]. Consistent with the above results, levels of uridine and hypoxanthine were lower in the model group than those in the control, indicating that HFD intake could lead the suppression of purine metabolism and pyrimidine metabolism, or the reduction of gut microbiota. AEE treated group showed recovery patterns of hypoxanthine and uridine. From these results, HFD might induce alterations in the metabolisms of purines and pyrimidine or gut microbiota, which could be attenuated by the AEE treatment. Linoleoyl ethanolamide was a fatty acid ethanolamide. There is no study about the relationship of linoleoyl ethanolamide with hyperlipidemia, and it would be interesting to investigate the biological function of linoleoyl ethanolamide in hyperlipidemic rats in future studies.

Compared with our previous fecal metabonomics study, lower number of metabolites was found in the cecal contents in present study [19]. Poor therapeutic effects caused by the administration method might have limited impact on the cecal contents metabolites, which might be the reason for finding few metabolites. Polakof et al reported that linoleic acid level in the cecal contents from HFD-fed rats was significantly higher than the control rats, which was consistent with the changes of linoleic acid in present study [46]. Surprisingly,

opposite change trends of linoleic acid were observed in previous fecal metabonomics studies [19]. It has been reported that oxidation products major from linoleic acid in feces were significantly increased in the HFD-induced atherosclerotic rats [43]. Oxidation process of linoleic acid might severely deplete itself, which could result in low level of linoleic acid in feces. In addition, there was a close relationship between linoleic acid metabolism and bacterial community [47]. Different microbiota composition in cecal contents and feces from control and HFD-fed rats might cause the difference in linoleic acid metabolism. Therefore,it was speculated that linoleic acid had different metabolic transformation process in cecal contents and feces, which might be the reason for heterogeneous results of linoleic acid. In HFD-fed rats, AEE treatment might affect linoleic acid metabolism or microbiota composition to regulate linoleic acid level in cecal contents and feces.

Recently, it has been reported that the bacteria in the gut interact extensively with the host through the metabolic exchange and co-metabolism of substrates and gut bacterial composition was closely linked to hyperlipidemia [4, 48]. In the present study, both PCoA and cluster analysis indicated that AEE treatment altered the structural composition of the cecal microbiota and reversed the dysbiosis caused by HFD. PCoA score plots and cluster analysis of the samples in AEE group showed tendencies similar to that of the control. Yet the microbial community was not completely restored in the rats after AEE treatment in present study. From the view of Shannon and Simpson indexes, AEE displayed positive effects on microbial diversity. The obtained results showed that HFD changed the abundance and diversity of the gut microbiota in rats. For example, the abundance of *Firmicutes* and *Euryarchaeota* increased and decreased, respectively. *Firmicutes* could absorb the calories in the diet and increase the fat storage in the body. Therefore, the improved rats' blood lipids in AEE treatment group may be related to the recovered abundance and diversity of the gut microbiota such as the reduction of *Firmicutes*.

Combination of LEfSe and statistical significance was employed to determine the features which might explain the differences among groups. Interestingly, the results of cladogram showed that *Methanobacteria* were observed in the control. Previous study proved that *Methanobacteria* can scavenge ammonia as substrates for the generation of methane and to increase the capacity of polysaccharide-metabolizing bacteria [49]. So the residing of *Methanobacteria* in the gut of the rats in the control group was beneficial for physiological functions. Results of LDA score showed a significant increase of phylum *Firmicutes* with major *Turicibacter* in HFD rats as compared to the control. Susanne et al. reported that the relative proportion of *Turicibacter* could be increased by the HFD in the C57BL/6 J mice, and *Turicibacter* had a strong positive correction with body weight gain and energy harvest [50]. Conversely, the relative abundance of *Turicibacter* was lower in the AEE treated rats, which might have beneficial effects on hyperlipidemia treatment by inhibiting energy absorption. In addition, *Staphylococcus* was significantly reduced in the AEE group in comparison with the model. It was reported that *Staphylococcus* infection in HFD-fed dogs could lead to the impairment of glucose tolerance through the damages of insulin secretion and insulin sensitivity [51]. It is known that there is a close relationship between hyperlipidemia and glucose metabolism [52]. Therefore, the reduction of *Staphylococcus* caused by AEE might improve glucose tolerance, which had benefits on hyperlipidemia treatment or reducing diabetes risk to keep host healthy.

Moreover, the correlations were observable between the cecal contents metabolites and microbiota, which could provide interactive functional information associated with AEE treatment. A great number of studies have confirmed the correlations between gut microbiota and metabonomics in HFD-treated animals [24, 53]. Our results showed that there was a possible link between the altered microbiota and metabolites in AEE-treated rats. However, the sophisticated mechanism between endogenous metabolites and microbes affected by AEE treatment has not been clearly elucidated. In Table 4, it was important to note that the effects of AEE treatment on some genera were changed parallel with those in the model group. There were two possible reasons for these results. First, gut microbiota contains some 10^{13}–10^{14} bacteria, each of them with their own unique sensitivity to drug. For example, eugenol is known to possess antimicrobial activity in a wide spectrum of bacteria from various levels of concentrations [54, 55]. It was speculated that the AEE treatment had diverse effects on gut bacteria such as bacteriostatic or bactericidal effects, which might result in the changes of genera in gut microbiota. Second, the different changes of endogenous metabolites may be other potential cause. Increasing evidences have indicated the significant interplay between gut microbiota and mammalian metabolism. In this study, AEE made a significant difference on cecal contents metabolites, and then changed metabolites might affect genera abundance. In future, more studies are needed to elucidate the interactions between AEE and specific genera.

It was important to note that this study had some limitations. First, this study did not compare the effects of AEE with its parent compounds, which could provide direct evidence to display the advantages of AEE. Second, the deep action mechanism among AEE, microbiota and metabolic pathways remains unknown. Further studies are

needed to be done to explicate the interactions of AEE with microbiota and metabolic pathways associated with hyperlipidemia.

Conclusions

LC–MS based metabolomics and 16S rRNA gene sequencing were combined to assess the effects of AEE on HFD-induced hyperlipidemia. The results showed that AEE treatment ameliorated not only cecal contents metabolism but also cecal microbiota composition in HFD-fed rats. The metabonomic analysis revealed that eight metabolites involved in purine metabolism, linoleic acid metabolism, glycerophospholipid metabolism, sphingolipid metabolism and pyrimidine metabolism were regulated by AEE treatment. AEE also normalized the HFD-induced alternations in the gut microbiota such as the reduction of *Staphylococcus* and *Turicibacter*. Furthermore, the correlation analysis revealed the possible link between the identified metabolites and gut microbiota. These findings indicated the regulation effects of AEE on cecal contents metabonomics profile and microbiota, which could provide new evidence to understand the possible action mechanism of AEE for hyperlipidemia treatment.

Methods

Reagents and materials

AEE (transparent crystal, purity: 99.5% with RP-HPLC) was prepared in Key Lab of New Animal Drug Project of Gansu Province, Key Lab of Veterinary Pharmaceutical Development of Agricultural Ministry, Lanzhou Institute of Husbandry and Pharmaceutical Sciences of Chinese Academy of Agricultural Science. The commercial kits for blood biochemical parameters were provided by Ningbo Medical System Biotechnology Co., Ltd. (Ningbo, China). MS-grade formic acid was supplied by TCI (Shanghai, China). Deionized water (18 MΩ) was prepared with a Direct-Q®3 system (Millipore, USA). MS-grade acetonitrile was purchased from Thermo Fisher Scientific (USA). Standard compressed rat feed and high diet feed (HFD) were supplied by Keao Xieli Feed Co., Ltd. (Beijing, China). Standard rat diet consisted of 12.3%lipids, 63.3% carbohydrates, and 24.4% proteins (kcal) and HFD (77.8% standard diet, 10% yolk power, 10% lard, 2% cholesterol and 0.2% bile salts) consisted of 41.5% lipids, 40.2% carbohydrates, and 18.3% proteins (kcal).

Animals and grouping

Forty-five male Wistar rats, weighing 180–200 g, were purchased from Lanzhou Veterinary Research Institute (Lanzhou, China). Rats were housed in plastic cages (size: 50 × 35 × 20 cm, 10 rats per cage) with stainless steel wire cover and chopped bedding. Rat feed and drinking water were supplied ad libitum. Light/dark regimen was 12/12 h and living temperature was 22 ± 2 °C with relative humidity of 55 ± 10%. After 2-week adaptation, rats were randomly separated into three groups (15 rats in each group with two cages, 7 or 8 rats per cage) and fed experimental diets for eight weeks. One group as control group was fed with standard diet, whereas the other two groups were fed with a high fat diet containing or not AEE (HFD and HFD plus AEE, respectively). AEE powder was added in HFD at dose of 850 mg/kg diet, and the approximate dose of AEE administered to rats was 43.5 mg/kg body weight in the experiment. The food intake of each group and body weights of individual rats were recorded weekly. A summary of study design used in this work was shown in Fig. 5.

Sample collection

At the end of experiment, rats were fasted for 10–12 h before blood sampling. Rats were euthanized with 1% pentobarbital sodium (intraperitoneal injection, 30 mg/kg), and then the blood samples were withdrawn from the heart into different vacuum tubes [56]. The blood in Na-heparin vacuum tubes were used for hematological

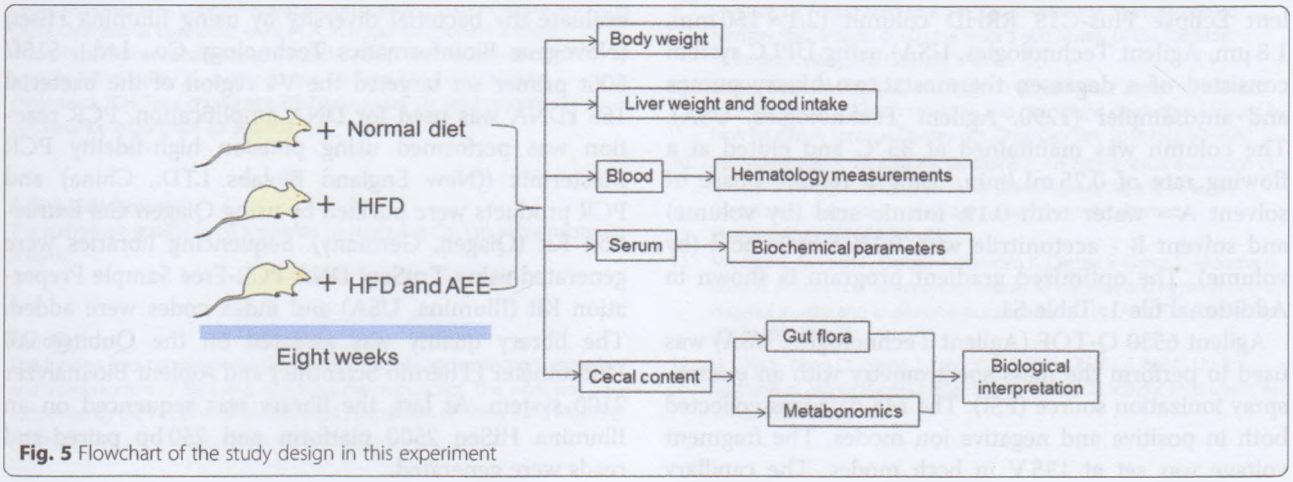

Fig. 5 Flowchart of the study design in this experiment

measurement which was performed in one hour. Blood in vacuum tubes without anticoagulant were centrifuged to obtain serum (4000 *g*, 4 °C for 10 min). Serum samples were stored at − 80 °C until biochemical analysis. Cecal contents were rapidly removed and frozen in liquid nitrogen, and then were stored at − 80 °C until processed.

Blood analysis

Whole blood was analyzed by BC2800-Vet (Mindray, China) to perform hematological measurements. Hematological parameters were consisted of white blood cell (WBC), lymphocyte (LY), monocyte (MONO), neutrophils (NEUT), red blood cell (RBC), PLT, hematocrit (HCT), mean corpuscular volume (MCV), red blood cell distribution width coefficient of variation (RDW-CV), mean platelet volume (MPV), and platelet distribution width (PDW). With the application of XL-640 automatic analyzer (Erba, Germany), serum samples were analyzed to measure the levels of biochemical parameters including total bilirubin (TB), direct bilirubin (DB), total protein (TP), albumin (ALB), globulin (GLOB), ALT, AST, ALP, LDH, CK, urea, creatinine (CREA), glucose (GLU), TG, HDL, LDL and TCH. For assessing lipid changes, the AI was calculated as followed: AI = (TCH - HDL)/HDL.

Sample preparation for metabonomics analysis

Cecal contents were lyophilized and then pulverized. 300 μL cold (− 20 °C) methanol was added into 50 mg cecal contents in 2 mL centrifuge tube. After vortex-mixing for 2 min, the mixture was treated with ultrasonic extraction and then centrifuged at 14,000 *g* for 15 min at 4 °C. After the supernatant was filtered through a 0.22 μm nylon filter, an aliquot of 2 μL was injected for analysis. Four QC samples prepared by mixing equal aliquots of cecal contents supernatant were inserted regularly in the analysis sequence.

Metabolic profiling data acquisition

Chromatographic separation was carried out on an Agilent Eclipse Plus-C18 RRHD column (2.1 × 150 mm, 1.8 μm, Agilent Technologies, USA) using UPLC system consisted of a degasser, thermostat,two binary pumps and autosampler (1290, Agilent Technologies, USA). The column was maintained at 35 °C and eluted at a flowing rate of 0.25 mL/min, using a mobile phase of solvent A - water with 0.1% formic acid (by volume) and solvent B - acetonitrile with 0.1% formic acid (by volume). The optimized gradient program is shown in Additional file 1: Table S1.

Agilent 6530 Q-TOF (Agilent Technologies, USA) was used to perform the mass spectrometry with an electrospray ionization source (ESI). The MS data was collected both in positive and negative ion modes. The fragment voltage was set at 135 V in both modes. The capillary voltages were set at 4.0 KV and 3.5 KV in positive and negative modes, respectively. The desolvation gas rate was set to 10 L/min at 350 °C with the use of drying gas nitrogen. The pressure of the nebulizer was set at 45 psig. Data was collected in centroid mode from 50 to 1000 *m/z* and the scan time was set at 1 spectra/second. MS/MS analysis was carried out to confirm the structure of the potential biomarkers. In addition, biochemical reactions and physiological roles related with endogenous metabolites were searched through KEGG and HMDB.

Metabonomic data analysis

The raw MS spectra were firstly converted to common data format (.mzData) by Mass Hunter Qualitative Analysis software (Agilent technologies, USA). Then peak alignment was carried out by XCMS program. Subsequently an integrated data matrix composed of compound mass, retention time, and peak intensities was generated. After normalization, the obtained data sets were imported into SIMCA-P V13.0 (Umetrics AB, Sweden) to perform PCA and PLS-DA. In order to avoid over fitting, PLS-DA models were validated by permutation test (with 200 permutations). The parameters of the PLS-DA models including R^2X, R^2Y, and Q^2Y were analyzed to ensure the model quality, and the R^2Y-, Q^2Y-intercepts of permutation test were examined and to avoid the risk of over-fitting. *VIP* values and loading-plots were applied to find potential biomarkers. A Wilcoxon Mann Whitney test with false discovery rate (FDR) limit equal to 0.05 was employed for univariate analysis. With VIP value above 1.0 and adjusted *P* value below 0.05, the candidate metabolites were considered to be potential biomarkers.

DNA extraction and sequencing of cecal microbiota

Total bacteria DNA was extracted from caecal contents by using PowerFecal™ DNA Isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) according to manufacturer's instruction. The 16S rRNA gene was analyzed to evaluate the bacterial diversity by using Illumina Hiseq (Novogene Bioinformatics Technology Co., Ltd.). 515f/806r primer set targeted the V4 region of the bacterial 16S rDNA was used for DNA amplification. PCR reaction was performed using phusion high-fidelity PCR Mastermix ((New England Biolabs LTD., China) and PCR products were purified by using Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on an Illumina HiSeq 2500 platform and 250 bp paired-end reads were generated.

Pairs of reads from the original DNA fragments were merged by using FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/). Sequencing reads was assigned to each sample according to the unique barcode of each sample. Chimeric sequences were removed using the USEARCH software and the microbial diversity was analyzed using the QIIME software (Version 1.7.0) with Python scripts (http://qiime.org/). OTUs were picked with a 97% similarity threshold. Alpha diversity and Beta diversity (both weighted and unweighted unifrac) were calculated with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3). Cluster analysis was preceded by PCoA using WGCNA package, stat packages and ggplot2 package in R software.

Statistical analysis

The results of the data were expressed as mean ± standard deviation (SD). The differences among experimental groups had been evaluated by one-way ANOVA with Fisher's least significant difference (LSD) test using the Statistical Package for Social Science program (SPSS 16.0, Chicago, IL, USA). The significance threshold was set at $P < 0.05$ for the test.

Additional file

Additional file 1: Table S1. Optimized gradient elution program of UPLC-Q-TOF/MS in cecal content metabonomic study. **Table S2.** OTU table summary of the samples. **Table S3.** Difference in relative abundanceof gut microbiotaatphylum level. **Figure S1.** TIC of cecal content samples in positive and negative modes. **Figure S2.** PCA score plot basedon the cecal content metabolic profiling in positive and negative modes. **Figure S3.** Rarefactioncurve of the cecal content samples.

Abbreviations

AEE: Aspirin eugenol ester; AI: Atherosclerosis index; ALB: Albumin; ALP, : Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CK, : Creatine kinase; CREA: Creatinine; DB: Direct bilirubin; ESI: Electrospray ionization source; FDR: False discovery rate; GLOB: Globulin; GLU: Glucose.; HDL: High-density lipoprotein; HFD: High fat diet; LDH: Lactate dehydrogenase; LDL: Low-density lipoprotein; LysoPC: Lysophosphatidylcholine; OUTs: Operational taxonomic units; PCA: Principal component analysis; PCoA: Principal coordinate analysis; PLS-DA: Partial least squares discriminant analysis; PLT: Platelet QC, quality control; TB: Total bilirubin; TCH: Total cholesterol; TCH: Total cholesterol; TG: Triglycerides; TP: Total protein; VIP: Variance importance for projection

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Authors' contributions

JYL and YJY designed and supervised the experiments. NM, XWL, XJK and conducted the experiment. SHL, ZQ and ZHJ prepared materials. JYL NM and YJY prepared the manuscript and reviewed the literature. All authors read and approved the final manuscript.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Pharmacokinetics of rectal levetiracetam as add-on treatment in dogs affected by cluster seizures or status epilepticus

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Abstract

Background: Levetiracetam can be used for seizure control alone or in combination with other antiepileptic medications. A previous study achieved the minimum targeted serum drug concentration after rectal administration of levetiracetam in healthy dogs. The purpose of the present study was to determine the pharmacokinetics of rectal LEV in dogs presented for cluster seizures or status epilepticus and potentially in treatment with other anti-epileptic drugs. Furthermore, preliminary information on response to this treatment as add-on to the standard treatment protocol is reported.

Results: Eight client-owned dogs were enrolled. Plasma levetiracetam concentrations (measured at 0, 30, 60, 90, 120, 180, 240, 360, 720, and 1440 min after drug administration) reached the minimum target concentration (5 μg/ml) at 30 min in all but one patient. At T1 (30 min) the mean concentration was 28.2 ± 15.5 μg/ml. Plasma concentrations remained above the targeted minimum concentration in all patients until 240 min and in 7/8 until 360 min. Six out of eight patients experienced no seizures in the 24-h period after hospitalization and were classified as "responders".

Conclusions: Minimum plasma levetiracetam concentration can be reached after rectal administration of 40 mg/kg in dogs affected by cluster seizures and status epilepticus and concurrently receiving other antiepileptic drugs. These preliminary results may encourage the evaluation of rectal levetiracetam as an additional treatment option for cluster seizures and status epilepticus in a larger number of dogs.

Keywords: Epilepsy, Pharmacokinetics, Neurology, Emergency

Background

Canine epilepsy is among the most common neurological diseases in dogs [1]. Cluster seizures (CS) are defined as the occurrence of two or more seizures within a 24-h period, with complete recovery of the state of consciousness in between; status epilepticus (SE) refers to seizure activity lasting for 5 min or longer or when there's no complete recovery of the state of consciousness between two seizure events [2]. CS and SE are potentially life-threatening neurological emergencies and are considered risk factors for spontaneous death or euthanasia of dogs affected by epilepsy [3–7]. As such, these conditions are a frequent reason for presentation to emergency veterinary services [8, 9].

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To date, first line therapy is intravenous or rectal administration of diazepam during the seizure event [10–12]. Unfortunately, not all dogs will respond to benzodiazepines and can experience refractory SE. Moreover, prolonged seizure activity is known to decrease the effectiveness of benzodiazepines in human medicine [13]. Levetiracetam (LEV), a pyrrolidone derivative, is a novel antiepileptic drug that was approved in the United States in 1999 for the oral treatment of partial onset seizures in humans [14]. Its mechanism of action is not fully understood, but it seems to differ completely from other antiepileptic medications (AEDs). LEV is thought to act by binding the synaptic vesicle protein 2A on the presynaptic terminal, thus modulating exocytosis of neurotransmitters [15]. Due to its favorable therapeutic profile, LEV has been increasingly used for seizure control either alone or in combination with other

first line AEDs in veterinary medicine [16]. In their study published in 2014, Peters and colleagues found a rapid rise in serum LEV concentrations associated with maintenance of values above the targeted minimum concentration up to 9 h after rectal administration of a LEV formulation in healthy dogs [17].

Based on these premises, the aim of this pilot study was to determine the pharmacokinetics of LEV administered per rectum in dogs presented for CS or SE and possibly already in treatment with other long-term AEDs. We hypothesized that LEV administered per rectum would achieve the targeted minimum plasma drug concentration in patients affected by CS and SE. Furthermore, we report the response to treatment as preliminary information on the potential association of LEV administered per rectum as an adjunct to standard treatment in patients referred for CS and SE.

Methods

Animals

The study was approved by the Bioethics Committee of the University of Turin (protocol #9834 dated 25/02/2016). The owners gave their written, informed consent to their dog's enrollment in the study. Client-owned dogs (minimum weight 20 kg) presented with CS or SE to the Veterinary Teaching Hospital (VTH), Department of Veterinary Science of Turin, between October 2016 and April 2017 were eligible for inclusion. SE was defined as a seizure event lasting more than 5 min or two or more seizures without complete recovery of consciousness in between. CS were defined as two or more seizures occurring within a 24-h period. Dogs were excluded if they were already in treatment with LEV for long-term seizure control or if further diagnostic tests indicated reactive seizures.

Study design

At the time of presentation to the VTH, seizure activity was immediately controlled by standard care comprising rectal administration of diazepam (at a dosage of 1–2 mg/kg if the dog was seizuring at presentation) followed by IV administration of phenobarbital (4–5 mg/kg q8h). As soon as possible after hospitalization, and always within 2 h from the presentation, LEV suspension (at a dosage of 40 mg/kg) was administered per rectum. The dosage was based on the results of a previous study [17]. A rigid, sterile, male dog urinary catheter (BUSTER Disposable Dog Catheter, Buster, Kruuse, Germany) was cut to 5 cm length and inserted approximately 3 to 4 cm into the rectum. A syringe was then connected to inject the drug. The catheter was flushed with air immediately after the injection to ensure the administration of the remaining portion of LEV in the catheter. After removal of the catheter from the rectum, the anus was held closed for 5 min to prevent drug expulsion. The procedure was performed by the same investigator (G.C.) in all patients.

Venous blood samples were obtained immediately before drug administration (T0), and at 30 (T1), 60 (T2), 90 (T3), 120 (T4), 180 (T5), 240 (T6), 360 (T7), 720 (T8), and 1440 (T9) min thereafter. Blood samples were collected in ethylenediaminetetraacetic acid tubes, and plasma was separated immediately after sampling by centrifugation at room temperature (3500 × *g*, 5 min) and then frozen at − 20 °C until analysis. Patients were assessed for signs of adverse reactions specifically attributable to LEV administration (decreased appetite and vomiting) by the same investigator (G.C.) at each time point and between the experimental time points by the intensive care unit veterinarians.

For the assessment of treatment efficacy, dogs were defined as "responders" if no further epileptic seizures occurred during the 24-h observation period between hospital admission and discharge; "non-responders" were dogs that experienced an additional epileptic seizure despite LEV administration in addition to the above-mentioned protocol in the 24-h period.

Levetiracetam suspension

Pure LEV powder (Levetiracetam European Pharmacopoeia Reference Standard, Sigma-Aldrich, Saint Louis, MO, USA) was purchased and mixed with sterile water to make a suspension with a LEV concentration of 200 mg/ml. This was done to reduce the volume of solution for rectal administration and minimize the risk of accidental evacuation of the drug. The suspension was formulated and replaced every month. LEV suspension was stored at room temperature away from direct light and always vigorously shaken to suspend the powder before administration.

Determination of plasma levetiracetam concentrations

Levetiracetam powder and all other reagents were purchased from Sigma-Aldrich. LEV was analyzed on a high-performance liquid chromatography (HPLC) system (Dionex Thermo Fischer Scientific, Sunnyvale, CA, USA) and separation was performed on a C18, 5 µm, chromatography column (Dionex Thermo Fischer Scientific) protected by a security guard precolumn. Chromatographic run was carried out at 35 °C for 20 min with a step gradient starting at 0 min with 95% solvent A (H_3PO_4 0.423% in water) and reaching 100% solvent B (acetonitrile) at 12 min. Detection was performed at $\lambda = 210$ nm. The limit of detection was 1 µg /ml. For LEV extraction, 500 µl of plasma were mixed with 10 µl of $HClO_4$ and 500 µl of methanol. The samples were then vortexed for 2 min and centrifuged at 17,000 × *g* for 5 min. Forty microliters of supernatant were then analyzed by HPLC. The unknown concentrations of LEV in samples were quantified by comparing

the signal to standard calibration curve ($R^2 = 0.9947$). The recovery percentage was 99.2 ± 4.9%.

Data analysis

Continuous variables, including patient age and weight at inclusion, were reported as median (minimum – maximum) [min – max]. Pharmacokinetic parameters were estimated by plotting LEV concentrations versus time. Data were analyzed using a Chromelion 6 Chromatography data system (Chromelion 6 Chromatography data system, Thermo Fischer Scientific), and statistical analysis was performed using GraphPad InStat 3.0 (GraphPad InStat 3.0, GraphPad Software, La Jolla CA, USA). Parameters were area under the curve (AUC), maximum concentration (C_{max}), time to maximum concentration (T_{max}), and half-life (t ½). Non-compartmental analysis was performed with AUC calculated using the linear trapezoidal method. The Shapiro-Wilk test showed normal distribution of the dataset; data were reported as mean ± standard deviation (SD).

Results

A total of 36 dogs were presented for CS or SE to the VTH between September 2016 and April 2017. Eight dogs met the inclusion criteria and were included in the study. The other 28 patients were excluded because: body weight less than 20 kg (16/28), no consent given by the owners for inclusion in the study (7/28), long-term oral LEV administration for seizure control (3/28), and diagnosis of reactive seizures (2/28).

Among the eight dogs included in the study, five were intact females and three were males (two intact and one neutered); the median age and weight at presentation were 75 months (range, 43–126) and 34 kg (range, 24–52), respectively. Detailed information on signalment and history are reported in an additional file (see Additional file 1). Blood work comprising complete hematology and biochemistry panel, bile acid stimulation test, and blood ammonia concentration resulted within normal limits. Four dogs were diagnosed with suspected idiopathic epilepsy based on signalment, history, and normal interictal neurological examination. Magnetic resonance imaging (MRI) and cerebrospinal fluid (CSF) analysis were available for only one patient and were unremarkable. Signalment, history, and abnormal interictal neurological examination aroused suspicion of structural epilepsy in the four other patients. In two of these cases a neoplastic lesion (suspected glioma) was confirmed by MRI investigation. A space-occupying lesion was suspected in the other two patients based on signalment and the findings of neurological examination. The neurological examination was performed by a board-certified neurologist (A.D.A.) or a neurology resident (G.C.) under supervision of the board-certified neurologist.

At the time of inclusion in the study, four out of eight dogs had been receiving phenobarbital (PB) therapy for long-term seizure control; two were concurrently receiving potassium bromide (KBr) and one patient was on treatment with Imepitoin. The remaining three dogs had not received any previous AED therapy (see Additional file 1). The patients receiving PB alone or in combination with KBr had been in treatment for longer than the period needed to achieve steady state of the drugs (14 days and 1–3 months, respectively). PB dosage varied from 2.6 to 6.3 mg/kg q12h, (median, 3.6 mg/kg q12h); the KBr dosage was 40 and 27 mg/kg q24h in each of the two dogs, respectively. Imepitoin was administered at a dosage of 15.7 mg/kg q12h.

Plasma LEV concentrations at the nine time points are shown in Fig. 1. At the first experimental time point (T1) the mean concentration was 28.2 ± 15.5 μg/ml ($n = 8$). At

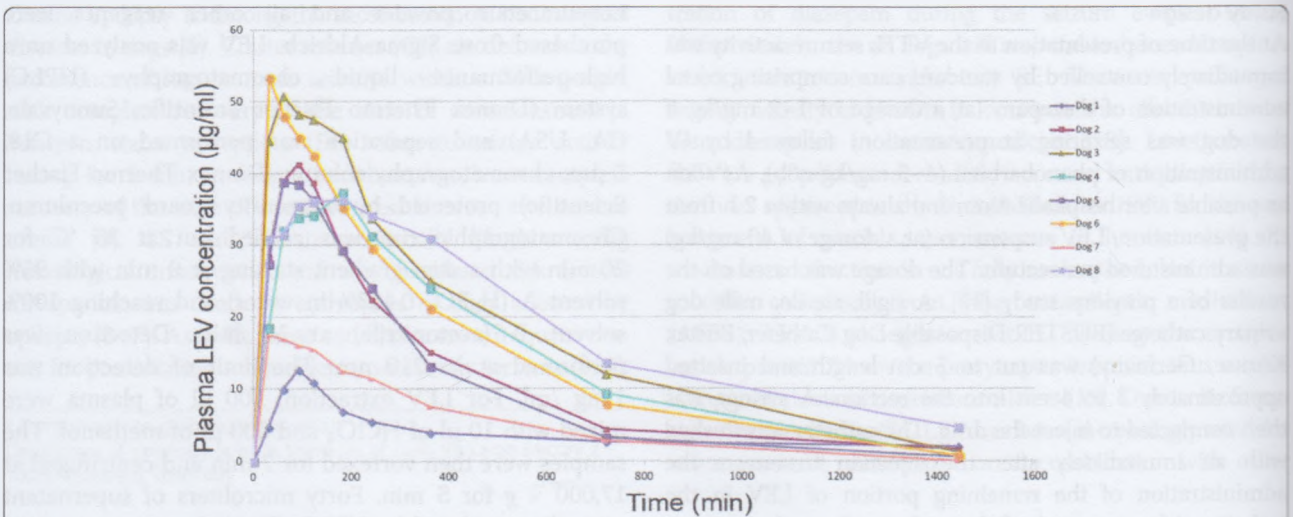

Fig. 1 Time course of plasma LEV concentration after rectal administration

this time point (T1), plasma LEV concentrations reached the minimum target concentration of 5 μg/ml in all but one patient, in which it was slightly lower than the target (4.7 μg/ml). Plasma LEV concentrations remained above the minimum target range in all patients until T6 and in 7/8 (88%) patients at T7.

The plot of plasma LEV concentration versus time showed a lower peak concentration and a more rapid decrease in LEV concentration over time in two patients. Pharmacokinetic analysis of the data from the eight dogs revealed a C_{max} of 36.0 ± 14.4 μg/ml, with a T_{max} of 90 ± 60 min. The t ½ was 251.7 ± 75.6 min and the AUC 227.8 ± 131.8 μg-h/ml.

Six out of eight patients (75%) experienced no further seizures during the 24-h observation period and between hospital admission and discharge. Two patients (25%), diagnosed with confirmed and suspected idiopathic epilepsy, respectively, and both with lower peak concentrations and a more rapid decrease in LEV concentration over time, were classified as "non-responders". They required further medications (constant rate infusions of diazepam in one and constant rate infusion of propofol in the other) for seizure control.

Considering the different outcomes, a post-hoc analysis was carried out with the patients grouped into "responders" ($n = 6$) and "non-responders" ($n = 2$). The results are shown in Table 1.

Discussion

To our knowledge, this is the first study to evaluate LEV concentration after rectal administration in dogs presenting with CS or SE and potentially receiving concurrent therapy with other AEDs for long-term seizure control. In line with the observations reported by Peters and colleagues [17], our results show that the targeted minimum plasma LEV concentration can be achieved with rectal administration of 40 mg/kg. In the majority of cases, plasma concentrations reached the minimum targeted concentration after rapid absorption, already at the first blood sample taken 30 min after administration of the drug. A therapeutic range of LEV specific for dogs has not yet been established. The values of 5–45 μg/ml typically employed in veterinary medicine are deduced from human medicine [18]. The therapeutic range is highly variable, however, even in human patients, and mainly in correlation with age [19].

Peters and colleagues highlighted the potential risk of lower LEV absorption after rectal administration if palpable fecal material is present in the rectum [17]. The lower values of C_{max} and T_{max} for the two dogs in our series may be associated with less absorption of the drug due to the presence of fecal material. Another possible explanation for the different results is the concurrent long-term administration of PB. Indeed, LEV undergoes predominant renal excretion as unchanged drug (47 and 58% in female and male dogs, respectively). The remaining percentage of the drug is metabolized as acid metabolites and hydroxylated metabolites by hydrolysis and oxidation, respectively. This latter route of degradation was found to be induced by PB in rats and dogs [20]. Further investigations on dogs confirmed that chronic PB administration alters the metabolism of LEV, resulting in lower concentrations and more rapid renal clearance of LEV when administered per os [21, 22]. In all studies performed in veterinary medicine, only 21 days of PB administration were proven sufficient to increase metabolism of LEV, so the chronic PB administration is an unlikely explanation for the results in these two dogs. Unfortunately, we did not check for the presence of fecal material since we wanted to replicate the conditions in which rectal administration is performed in clinical settings. While this could be the most plausible explanation, we are unable to determine whether the lower drug absorption was due to any fecal material potentially present at the time of administration. This issue represents a limitation of the present study. Further studies are needed to evaluate the pharmacokinetics of rectal LEV in patients concurrently receiving PB, while excluding the confounding factor of feces present in the rectum. If this assumption is confirmed, it could also be interesting to assess the feasibility and safety of higher doses of LEV administered per rectum in patients under PB therapy.

One of the two dogs classified as "non-responders" had been diagnosed with idiopathic epilepsy at the time of inclusion in the study. According to the revised definition of pharmacoresistant epilepsy issued by the International League Against Epilepsy in human medicine in 2010 [23], this patient can be classified as pharmacoresistant, and so this condition could explain the patient's non-responsiveness to treatment. Since seizure

Table 1 Pharmacokinetics parameters

	C_{max} (μg/ml)	T_{max} (min)	AUC_{0-t} (μg-h/ml)	t ½ (min)
"Non-responders" ($n = 2$)	12.7 and 17.53	90 and 30	89.63 and 105.24	153 and 249
"Responders" ($n = 6$)	43.02 ± 7.27	100.2 ± 64.8	337.94 ± 83.41	268.6 ± 75.8

No adverse effects specifically attributable to LEV administration were noted at any time point

frequency was not recorded by the owner of the second non-responder patient diagnosed with suspected idiopathic epilepsy, it is impossible to establish whether this dog can be classified as pharmacoresistant as well. If LEV efficacy can be demonstrated in a larger number of cases, achievement of the targeted minimum LEV plasma concentration with rectal administration in epileptic dogs might allow at-home use of this formulation for better seizure control. The usage of IV/oral LEV in so-called "pulse treatment" for cluster seizures is well known [16, 18]. Nevertheless, in dogs experiencing seizures, the administration of oral medications may be delayed by the post-ictal phase, potentially leading to further seizure events. The rectal route of administration would avoid this delay and improve seizure control.

In our study, we formulated LEV suspension at a concentration of 200 mg/ml to reduce as much as possible the volume of medication introduced into the rectum, thus preventing induction of defection and subsequent accidental expulsion of the drug. The LEV suspension was made using pure LEV powder for scientific reasons. We also made LEV suspension from commercially available LEV tablets and found no differences in the chemical purity of the two formulations (data available from the authors on request).

The main limitations of the present study are the small patient series, the concomitant use of other AEDs, and the absence of a control group of patients for comparison. The designation of "responder" and "non-responder" was in reference to the combination of medications administered, and therefore it is not possible to discern any potential effect of rectal LEV administration. Therefore, we cannot conclude that rectal LEV is effective in preventing the onset of further seizures in patients with CS or SE. Nonetheless, these preliminary pharmacokinetic data are promising and are consistent with those reported by Peters and colleagues. Given the postulated enhancement of the anticonvulsive effects of benzodiazepines and the lack of side effects, such as cardiac and respiratory depression typical of other AEDs, [24] LEV can offer a potentially useful add-on to the treatment of seizure activity in dogs once its efficacy has been confirmed in a greater number of cases. Further studies are needed to confirm or confute our preliminary hypothesis. A future area of focus of this project is to better evaluate the efficacy of rectal LEV in a larger number of cases.

Conclusions

In conclusion, our findings show that targeted minimum plasma LEV concentration can be reached after rectal administration of 40 mg/kg in dogs with CS or SE. These preliminary results, if confirmed, may allow for the use of rectal LEV as an additional treatment option for CS and SE in dogs.

Abbreviations

AEDs: Antiepileptic medications; AUC: Area under the curve; C_{max}: Maximal concentration; CS: Cluster seizures; CSF: Cerebrospinal fluid; HPLC: High performance liquid chromatography; KBr: Potassium bromide; LEV: Levetiracetam; MRI: Magnetic resonance imaging; PB: Phenobarbital; SE: Status epilepticus; T_{max}: Time point at which C_{max} is measured; VTH: Veterinary teaching hospital

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Authors' contributions

GC, ADA, and RO designed the experiments. GC, GG1, SA, GG2, LL, ED, IB performed the experiments. GC, RO, GG1, ADA analyzed the results. GC, RO, and ADA wrote the paper. All authors have critically revised and approved the manuscript. Acquisition of funding: ADA.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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A randomized, controlled, single-blinded, multicenter evaluation of the efficacy and safety of a once weekly two dose otic gel containing florfenicol, terbinafine and betamethasone administered for the treatment of canine otitis externa

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Abstract

Background: Otitis externa is a common problem in small animal practice. Compliance with daily treatment is a major cause of treatment failure. The hypothesis tested is that a novel otic gel applied to the ear canal twice with a one-week interval is as efficacious as a daily otic suspension in the treatment of canine otitis externa. The study included 286 privately owned dogs with otitis externa.

In this single blinded randomized study, enrolled dogs received either an otic gel containing 1% florfenicol, 1% terbinafine and 0.1% betamethasone acetate twice with a one-week interval or a suspension containing hydrocortisone aceponate, miconazole and gentamicin daily for 5 days. Ears were cleaned with saline prior to administration of the first dose of medication. Dogs were evaluated at day (D) 0, 7, 28 and 56 with an otitis index score (OTIS-3), otic culture and cytology, pain and pruritus, and overall response to treatment (owner and investigator evaluation). Outcome measures were improvement of the OTIS-3 and number of dogs in clinical remission at each time point.

Results: OTIS-3 decreased significantly ($p < 0.0001$) by 63 and 64% for the otic gel and by 63 and 61% for the suspension on D28 and D56 respectively. There was no significant difference between groups at any time point with regard to clinical success, pain, pruritus, overall assessments or otic cytology and culture. The treatment response was considered excellent or good by approximately three quarters of both the clinicians and Owners. Otitis recurrence at D56 was seen in 11% of both groups. Adverse events attributable to the ear medications were not noted.

Conclusions: Administering an otic gel twice at a one-week interval is an effective, safe and convenient way to treat canine otitis externa.

Keywords: Otitis externa, Otic gel, Clinical efficacy, Clinical safety, Florfenicol, Betamethasone, Terbinafine

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Background

Otitis externa is one of the more common presenting complaints in small animal practice [1]. The inflammation of the external auditory canal may be due to a number of causes such as hypersensitivities, endocrinopathies, parasites and foreign bodies [2, 3]. More than 50% of patients with atopic dermatitis exhibit otitis externa [4]. Predisposing factors include swimming, other causes of increased moisture in the ear canal or conformational factors such as pendulous pinnae [1, 3]. The consequence of the resultant inflammation and associated decreased ear canal lumen is almost invariably an infection with bacteria and/or yeast organisms [3, 5]. Most topical ear medications on the market therefore contain a combination of antibiotic, antimycotic and anti-inflammatory agents, typically administered once daily [6]. Such products are typically packaged in multi-dose presentations, increasing the potential risk of cross-contamination between ears. In addition to the lack of diagnosis and treatment of the underlying disease, the other major problem treating dogs with otitis is owner and patient compliance [7, 8]. Administration of ear medications into a swollen and often painful auditory canal is a procedure frequently disliked equally by the owner administering the medication and the patient receiving it [8]. In a recent study, formulations requiring infrequent administration and ear cleaning improved the overall Quality of Life for pet owners and their dogs to a greater extent than those requiring daily administration [9].

Increasingly, multi-resistant bacteria make treatment of infections in veterinary medicine more difficult [10–13]. One of the factors recognized to increase the risk for development of such multi-resistant bacterial isolates is repeated exposure to low concentrations of antibiotics [14, 15], such as may occur with poor compliance with administration of a topical antibiotic. Ear medication that reliably maintains an above therapeutic concentration of active ingredients in the ear canal without relying on daily administration by the pet owner could thus be of great benefit to both dogs with otitis externa and their owners.

The study reported here compared the clinical efficacy and safety for otitis externa of an otic gel formulated in a single-use tube administered twice at a one-week interval with a more typical otic suspension administered daily as per the manufacturer's recommendation, with the objective of demonstrating that the otic gel was non-inferior to the suspension.

Methods

Study design

In this randomized, single-blinded, positive-controlled, multicenter field study, dogs received either a viscous otic gel (Osurnia™, Elanco Animal Health, Greenfield, IN, USA) twice with a 1 week interval or an otic suspension (Easotic®, Virbac, Carros, France) daily for 5 days after an initial ear cleaning. Dogs were reevaluated at days 7, 28 and 56 after inclusion. The study was performed in accordance with the VICH GL9 (Good Clinical Practices). This manuscript was prepared after consultation of the checklist of the extension of the CONSORT statement for reporting of non-inferiority trials.

Study objects

Dogs with clinical signs of otitis externa, an otitis index score (OTIS-3) of at least 5 and cytologic evidence of bacteria or yeast were included in the study.

Inclusion, exclusion and withdrawal criteria

To be eligible for inclusion, dogs were required to be a minimum of 8 weeks of age, of any breed, weight, sex or neuter status. They were excluded due to otic foreign bodies or parasites, if intended for breeding, or if they had been treated with either systemic or topical antimicrobial/antifungals, ciclosporin or anti-histamines within the last 2 weeks, with ear cleaners or analgesic agents within the last week. Cases treated with systemic or topical anti-inflammatories (i.e. corticosteroids or Non-Steroidal Anti-inflammatory Drugs) within the last 28 days were not eligible for inclusion. Staff-owned or animals enrolled in other clinical studies within previous 3 months were not eligible for inclusion.

Similarly, dogs were not permitted into the study if they showed clinical signs of diseases which would interfere with the evaluation of the response. Lastly dogs in which the tympanum was ruptured or still not visible after an initial ear cleaning were also not included in the study. Withdrawal during the study occurred with adverse events that required intervention that could impact the study evaluation, lack of efficacy, administration of prohibited concomitant therapy, owner compliance or any other documented reason.

All study participants were required to sign an Owner Informed Consent prior to enrollment.

Randomization and blinding

Dogs were randomly allocated to the two treatment groups in a 1:1 ratio in blocks of four using the SAS/STAT® procedure PLAN (SAS, Cary, NC, USA). A separate randomization list was prepared by the statistician for each participating center and provided to the dispensers in the form of sealed numbered envelopes, each containing the treatment allocation according to order of inclusion.

Due to the obvious difference in treatment protocols, Owner blinding was not possible. In each center, a designated person, the "dispenser", was responsible for the allocation of the dogs to the treatment groups and the administration of the treatments on D0 (and in the group

treated twice with otic gel additionally on D7). Prior to each examination by the clinician, the dispenser recorded Owner answers regarding the clinical response, adverse effects, concurrent medications and other clinically relevant information, confirmed the Owner compliance, and prepared the Owner for the visit with the examining clinicians, to assure the continued blinding of the latter and gave relevant discharge instructions.

Intervention

Prior to inclusion, ear swabs were taken from each affected ear to collect material for culture and cytology. Subsequently, all dogs had their ears cleaned with physiologic saline to remove otic debris and permit visual evaluation of the tympana. Briefly this procedure consisted of filling the ear with warm saline solution, massaging the ear canal and wiping the entrance to the canal out with cotton wool. Dogs could be sedated for ear cleaning, if the ear was too painful to allow cleaning while conscious. Before discharge, each affected ear of dogs in the otic gel group received final formulation of 1 ml of a viscous gel (Osurnia, Elanco Animal Health, Greenfield, IN, USA) containing 1% florfenicol, 1% terbinafine and 0.1% betamethasone acetate. Affected ears of dogs in the control group were treated with one pump (delivering 1 ml) of a suspension (Easotic, Virbac, Carros, France) containing 1.11 mg/ml hydrocortisone aceponate, 15.1 mg/ml miconazole and 1505 I.U./mL of gentamicin. Owners of dogs in the control group were sent home with instructions to administer the otic suspension once daily for four more days. Dogs were reevaluated after 7 days and dogs in the otic gel group were treated again. Ear cleaning was not repeated in either group at D7. These treatment protocols are in accordance with the package inserts for the two products.

Clinical evaluation

Prior to cleaning of the ears on D0, an otoscopic examination was performed and the OTIS-3 determined. The total score was the sum of the scores for erythema, edema/swelling, erosion/ulceration and exudate, each graded between 0 and 3 (total range 0 to 12) [16]. In bilateral cases, the ear with the higher score was selected to be evaluated throughout for all parameters. If both ears had the same score, the right ear was evaluated throughout. OTIS-3 was determined without further ear cleaning, on D7, D28 and D56.

Overall assessments of response to treatment were performed by the Owners and Investigators at D7, D28 and D56 (excellent, good, moderate or poor; see Table 1). Investigators evaluated pain at each visit using a Numerical Rating Scale ranged 0–3 (0, none; 1, not painful on palpation but spontaneous head shaking; 2, not painful when pinna is raised but painful on palpation of base of ear; 3, painful when pinna is raised). Owners evaluated pain at each visit using a Visual Analog Scale (VAS) with the leftmost end marked 'The ear is not painful' and the rightmost end marked 'The ear is extremely painful'. Pruritus was assessed by Owners at each visit using a VAS with the leftmost corner underlined by the statement "The ear is not itchy" and the rightmost end with the statement 'The ear is very itchy all the time'.

Table 1 Investigator and Owner Overall Assessment Scales

	Investigator	Owner
Excellent	Clinical signs of the ear evaluated during the first examination have completely disappeared	My dog's ear condition has completely recovered as compared to before treatment
Good	Clear amelioration of the clinical signs of the ear evaluated compared to initial examination	My dog's ear condition has clearly improved compared to before treatment
Moderate	Slight amelioration of the clinical signs of the ear evaluated compared to initial examination	My dog's ear condition has responded only slightly to treatment
Poor	Worsening or no change of the clinical signs of the ear evaluated compared to the initial examination	My dog's ear condition has deteriorated or not changed compared to before treatment

Microbiological evaluation

At D0, slides were prepared for in-house cytology using each clinic's standard staining technique. These were examined under low magnification to find an area of interest, with oil then applied and the area was evaluated for presence of microorganisms and inflammatory cells. If the cytology revealed bacteria or yeast, further samples for cytology and culture were sent to a central laboratory (Idexx Laboratories, Ludwigsburg, Germany). Presence of yeast, bacteria and neutrophils was evaluated semi-quantitatively. For neutrophils, a score of 1 was assigned where no neutrophils were seen, 2 for 1–10, 3 for 11–20 and 4 for > 20 within five microscopic fields under × 600 magnification. For yeasts and bacteria, a score of 1 was given when no organisms were seen, few 2 (yeasts < 2, bacteria < 5), moderate 3 (yeasts 3–4, bacteria 5–25) and a high number of organisms recorded as 4 (yeasts > 5, bacteria > 25) based on five microscopic fields under × 600 magnification.

On D28 and D56 additional specimens were obtained for cytology and culture and sent to the central laboratory.

Minimum inhibitory concentrations

All bacterial and fungal isolates from ear swabs taken both before and after treatment (D28 and D56) from cases treated with the otic gel were supplied to a microbiological testing laboratory (Don Whitley Scientific, Shipley, United Kingdom). The minimum inhibitory

concentrations ($MIC_{50/90}$) against all bacterial and fungal isolates for florfenicol and terbinafine was determined using standardized broth microbiology as described by the Clinical and Laboratory Standards Institute (CLSI). Isolates of *Staphylococcus pseudintermedius* and *Staphylococcus aureus* were also screened for methicillin resistance.

Outcome measures for efficacy

The primary outcome measure was the percentage reduction in OTIS-3 at D28 compared to D0. Secondary outcome measures included the percentage of dogs with an OTIS-3 ≤ 3 (considered a clinical success) at D28 and D56; the percentage reduction in OTIS-3 at D56 compared to baseline; the overall assessments by the Owners and Investigators at D28 and D56; the number of dogs with an OTIS-3 ≥ 5 by D56 (considered relapses if the score at D28 was ≤ 3); the decrease of bacterial or fungal counts on cytology at D28 and D56; the frequency (percentage) of dogs with either a bacteriological or fungal response at D28 or D56 (defined as absence of the microorganism isolated at D0); decrease in pain assessed by the Investigator at D28 and D56; decrease in pain and pruritus assessed by the Owner at D28 and D56; and the speed of response assessed by reduction in OTIS-3 at D7.

Statistics

An intention to treat (ITT) analysis with the last observation carried forward was performed. Patients completing the study without major protocol deviations were included in a secondary per protocol analysis (PP). Group means were compared between the two groups using an analysis of covariance (ANCOVA) model with the treatment group and baseline response as model effects. The influence of covariates was assessed by a second set of ANCOVA models with body weight, exudate type, chronicity, recurrence, duration, prior treatment, and type of infection as model effects. All calculations were performed using SAS® Version 9.2.2, SAS® Prox Mixed and SAS® Proc Glimmix were used for the ANCOVA and the GLM models (SAS, Cary, NC, USA).

The mean and standard deviation (SD) of the percentage improvement in a previous unpublished pilot study was 66 and 28 respectively. With a non-inferiority margin of 15% (which was considered clinically relevant) and a minimal power of 80%, the appropriate number of study objects was calculated at 100 dogs per group.

Safety evaluation

Clinical safety of the product was evaluated through the reporting of Adverse Events (defined as any observation in an animal which was unfavorable and unintended and occurred after the use of either product) throughout the study period until D56. Blood samples for hematology and clinical chemistry were collected from all dogs at D0 prior to enrolment and at D28. Samples could be collected at other timepoints at the Investigator's discretion.

Results

Study objects

Thirty first opinion veterinary practices in France, Germany and the United Kingdom enrolled a total of 286 dogs in the study between April and September 2012. All dogs were considered for clinical safety, but one dog was excluded from all demographic and efficacy analyses as the Owner inadvertently retained both copies of the signed consent form. Demographics of the remaining 285 dogs (ITT population) are described herein. One hundred fifty-five males (51 neutered) and 130 female (68 neutered) were included for demographics and efficacy analyses. At the time of enrolment, dogs ranged in age from 10 weeks to 16.5 years with a mean (± SD) of 6.0 (± 3.8) years. The most common breeds represented were Labrador Retrievers (9%), Cocker Spaniels (9%), Golden Retrievers (6%) and Cavalier King Charles Spaniels (6%). Baseline demographics and disease characteristics are summarized by group in Table 2.

There was no significant difference in any demographic or disease characteristic between groups.

As the results of the ITT and PP analyses were similar, only the results of the ITT analysis are listed and discussed below for efficacy related outcomes.

Clinical evaluation

The mean OTIS-3 and their standard deviations are listed in Table 3.

Table 2 Baseline Demographics

Demographic	Otic Gel	Otic Suspension	*P*-value
Age in years (mean ± SD)	6.2 ± 3.8	5.8 ± 3.7	0.46
Number of Male/Female (%)	55/45	53/47	0.72
Weight in kg (mean ± SD)	21.9 ± 14.4	22.8 ± 14.8	0.62
Bilateral otitis	103 (70%)	101 (74%)	0.51
Ceruminous exudate	108 (73%)	96 (70%)	0.60
Purulent exudate	40 (27%)	41 (30%)	
Acute/subchronic/chronic	42 (28%), 87 (59%), 19 (13%)	30 (22%), 90 (66%), 17 (12%)	0.42
Number of days since onset (mean ± SD)	18.8 ± 22.8	18.6 ± 22.5	0.73
Previously treated	13 (9%)	17 (12%)	0.34
Recurrent	46 (31%)	46 (34%)	0.90
Culture yeast only (%)	25	27	0.89
Culture bacteria only (%)	22	20	
Culture yeasts and bacteria (%)	39	42	

Table 3 Mean OTIS-3 ± standard deviation of dogs treated with otic gel or otic suspension

Criterion	Visit	Otic gel ($N = 148$)	Otic suspension ($N = 137$)	*P*-value
OTIS-3	D 0	6.8 ± 1.6	6.8 ± 1.6	0.7490
	D 7	3.6 ± 1.7	3.0 ± 1.9	0.0027
	D 28	2.6 ± 2.2	2.6 ± 2.2	0.7992
	D 56	2.5 ± 2.4	2.7 ± 2.4	0.3532

There was a significant improvement in OTIS-3 in both groups after 28 days ($p < 0.0001$). The mean OTIS-3 stayed low until D56 in both groups. The OTIS-3 decreased on average by 62.5 and 63.6% for the otic gel and by 63.4 and 60.5% for the otic suspension on D28 and D56 respectively. Using the non-inferiority margin of 15%, the 95% confidence interval for the difference in percent reduction at D28 (otic suspension minus otic gel) had to be completely below 0.15 × 63.4% i.e. 9.5%. The Confidence Interval was calculated to be − 6.1 to 7.9%, so the primary endpoint of non-inferiority at D28 was concluded.

At D7, there was a significant difference between the two groups for total OTIS-3 ($p = 0.0027$) and the percentage reduction in OTIS-3 ($p = 0.0003$) in favour of the otic suspension. However, no significant difference was seen in the proportion of cases considered a clinical success by the Investigator at D7 (Table 4).

Approximately three quarters of clinicians and Owners reported a good to excellent response of otitis externa to the otic gel administration after D28 (Table 5), which was not significantly different to the suspension.

At D56, there were no differences between the group treated with the gel and that with the suspension in owners and investigators overall assessment scores. No significant differences in pain or pruritus scores were observed between the two groups at D28 or D56 ($p > 0.1860$; Table 6).

However, the owners reported a significant difference in pruritus at D7, in favor of the suspension ($p = 0.0232$). Relapses were recorded at D56 in 11 dogs (11%) treated with the otic gel, and in 10 dogs (11%) treated with otic suspension; there was no significant difference between the groups. Subsequent exploratory analyses did not identify any difference in clinical success at any timepoint between groups dependent on the weight of the dog ($p > 0.18$ across all weight bands and time points), chronicity of disease ($p > 0.1727$), recurrence ($p > 0.0947$), previous treatment ($p > 0.2025$) or type of exudate ($p > 0.0638$).

Table 4 Number (Percentage) of dogs reported as clinical treatment success[a] when treated with an otic gel or an otic suspension

Visit	Otic gel	Otic suspension	*P*-value
D7	80 (54%)	84 (61%)	0.2319
D 28	109 (74%)	98 (72%)	0.6926
D 56	106 (72%)	91 (66%)	0.3705

[a]Clinical success was defined as an OTIS-3 ≤ 3

Table 5 Clinician (owner in parentheses) overall assessment of treatment response (in %)

	D 28		D 56	
Response assessment	Gel	Suspension	Gel	Suspension
Excellent	29 (34)	31 (42)	39 (44)	38 (49)
Good	45 (49)	40 (42)	32 (36)	28 (24)
Moderate	19 (10)	20 (11)	18 (7)	22 (17)
Poor	7 (5)	8 (4)	11 (12)	12 (9)
Missing	1 (1)	1 (1)	1 (1)	1 (1)
p-value	0.9901 (0.2692)		0.6169 (0.7980)	

Microbiological evaluation

On D0, 40% of the overall study population had positive otic cultures for both bacteria and yeast, 26% had only yeast, 21% only bacteria and the remainder were culture negative; staphylococci of the *Staphylococcus pseudintermedius* group were most frequently cultured (47%), followed by *Pseudomonas aeruginosa* (11%), streptococci (11%) and enterococci (5%); in 18% of the dogs more than one bacterial species was identified. Of the proportion of the study population in which yeast was cultured, the most frequently identified organism was *Malassezia pachydermatis* (96%). There was no difference in distribution between groups for either bacteria or yeast species at D0.

The cytology counts for bacteria, fungi and neutrophils decreased in both groups, with no significant difference seen between groups (Table 6).

The overall bacteriological response (i.e. elimination of pathogens identified at baseline culture) as assessed by repeat of bacterial culture, was 60% and 49% for the gel at D28 and D56 respectively, compared to 60% and 55% for the otic suspension. For the fungal response, 76% and 65% of cases treated with the otic gel had responded at D28 and D56 respectively, in comparison to 69% and 55% for the otic suspension. None of these were significantly different (Table 6).

When clinical success (OTIS-3 ≤ 3 at D28) was considered for cases with different bacterial species, no significant differences were found between the products for any species. For *S. pseudintermedius*, the clinical success rate was 75% and 72% at D28 for the otic gel and the suspension respectively. For *P. aeruginosa*, the clinical success rates were 47% and 50% respectively for the otic gel and the otic suspension at D28.

Minimum inhibitory concentrations

The MIC data demonstrated that florfenicol was active against all bacterial groups, with MIC_{50} in the range 2 to

Table 6 Mean scores of all dogs for cytology scores, microbiological responses, pain and pruritus scores

	D0			D7			D28			D56		
	Gel	Suspension	*p*-value	Gel	Suspension	*p*-value	Gel	Suspension	*p*-value	Gel	Suspension	*p*-value
Cytology (Mean +/− SD)												
Bacteria	1.7 +/− 0.8	1.7 +/− 0.8	0.7479	n/a	n/a	n/a	1.1 +/− 0.9	0.9 +/− 0.7	0.2160	1.1 +/− 1.0	0.9 +/− 1.0	0.2233
Yeasts	2.1 +/− 0.8	2.2 +/− 0.8	0.3957	n/a	n/a	n/a	1.3 +/− 0.8	1.5 +/− 0.8	0.1077	1.5 +/− 0.9	1.5 +/− 0.9	0.4040
Neutrophils	1.2 +/− 0.4	1.3 +/− 0.7	0.6431	n/a	n/a	n/a	0.5 +/− 0.7	0.4 +/− 0.8	0.3488	0.5 +/− 0.7	0.4 +/− 0.9	0.2335
Bacteriological Response (%)	n/a	n/a	n/a				60	60	1.0000	49	55	0.4501
Fungal Response (%)	n/a	n/a	n/a				76	69	0.3318	65	55	0.1824
Investigator Pain Score (est. +/− SE)	1.77 +/− 0.07	1.69 +/− 0.07	0.4406	0.45 +/− 0.05	0.55 +/− 0.05	0.2371	0.38 /− 0.06	0.46 +/− 0.06	0.2967	0.42 +/− 0.06	0.54 +/− 0.07	0.1860
Owner Pain VAS (est. +/− SE)	31.4 +/− 2.8	31.1 +/− 2.9	0.9499	8.3 +/− 0.8	6.8 +/− 0.7	0.1437	3.9 +/− 0.5	3.3 +/− 0.4	0.3984	3.8 +/− 0.5	4.1 +/− 0.6	0.7676
Owner Pruritus VAS (est. +/− SE)	50.2 +/− 3.2	52.5 +/− 3.4	0.6334	10.8 +/− 1.1	7.8 +/− 0.8	*0.0232*	5.0 +/− 0.6	4.3 +/− 0.6	0.423	5.1 +/− 0.8	5.2 +/− 0.8	0.9208

Est. Estimated, *S.E.* Standard error, *VAS* Visual analog scale, *SD* Standard deviation; Entries in *italic* font are statistically significant

16 μg/ml except for *P. aeruginosa* (> 128 μg/ml). Due to low numbers of isolates for other pathogens, the MIC_{90} could only be calculated for *S. pseudintermedius* (8 μg/ml), *Streptococcus canis* (2 μg/ml) and *P. aeruginosa* (> 128 μg/ml). One hundred and two (102) *S. pseudintermedius* isolates were identified, of which 2 were identified as methicillin resistant on the basis of oxacillin zone diameter. Of 4 *Staphylococcus aureus* isolates, 2 were identified as methicillin resistant on the basis of cefoxitin zone diameter. All isolates identified as methicillin resistant exhibited florfenicol MICs within the same range as methicillin susceptible isolates of the corresponding species.

The only fungal species with greater than 10 isolates was *M. pachydermatis*. The MIC for terbinafine was in the range 0.125 to > 64 μg/ml, with an MIC_{90} of 2 μg/ml.

In 59% of cases, there was no change in the MIC for florfenicol between baseline and D28 of any organism identified in an individual ear. In 28% of cases, there was an increase of MIC by one dilution, with a decrease by one dilution in the remainder (13%). For terbinafine, there was no change in MIC between baseline and D28 for 55.5% of Malassezia cases, with 28% having an increase by one dilution, and 16.5% having a decrease by one dilution.

Safety evaluation

A total of 100 clinical signs relating to 80 adverse events were recorded in 30 of the dogs treated with the otic gel and 32 of the dogs treated with the suspension. These are summarized by System Organ Class in Table 7.

Cutaneous signs, such as generalized pruritus (rather than local to the ear) were the most common and observed in approximately one third of dogs showing adverse events, followed by slightly less dogs showing gastrointestinal signs. The majority of these adverse events were likely related to an underlying disease process such as atopic dermatitis. There was no difference in occurrence for any adverse event

Table 7 Clinical signs seen with administration of otic gel or suspension in dogs with otitis externa

System Organ Class	Otic Gel (*n* = 148)	Otic Suspension (*n* = 138)	*P*-value
Behavioral disorders	0	2	0.2328
Blood and lymphatic system disorders	3	0	0.2509
Digestive tract disorders	15	10	0.4315
Ear and labyrinth disorders	7	4	0.5516
Eye disorders	1	6	0.0617
Hepato-biliary disorders	1	0	1.0000
Mammary gland disorders	0	1	0.4825
Musculoskeletal disorders	1	3	0.3585
Neurological disorders	1	0	1.0000
Renal and Urinary disorders	0	1	0.4825
Reproductive system disorders	0	1	0.4825
Respiratory tract disorders	0	1	1.0000
Skin and appendages disorders	16	18	0.6105
Systemic disorders	5	2	0.4551
Any clinical sign	51	49	0.9204

between groups ($p = 0.9204$). There was no recognizable association between treatment and adverse events.

Three dogs treated with the otic gel showed clinically severe adverse events, none of those was considered to be related to medication due to the lack of temporal association and clinical signs observed. One dog had intestinal obstruction by peach stones 7 weeks after treatment, one a septic abdomen 6 weeks after treatment and the last one underwent exploratory surgery 7 days after inclusion in the study and showed gastrointestinal neoplasia. The first dog recovered uneventfully after surgery, the other two dogs were euthanized.

No changes in body weight, or clinically significant changes from baseline were seen in hematology or serum chemistry variables for either the gel or the suspension. There were no clinical pathology changes which could be considered of either hyper- or hypoadrenocorticism.

Discussion

In this study, in a population of dogs displaying typical characteristics of otitis externa, administration of an otic gel twice 1 week apart demonstrated similar efficacy and clinical safety to treatment with a conventional daily suspension for most endpoints. Only for the secondary endpoint speed of response (in terms of absolute and percentage reduction in OTIS-3 and Owner pruritus VAS) was the daily treatment significantly better than the otic gel. However, in another recently published study [9] using the otic gel, the percentage improvement in pruritus was considered to be better with the gel in comparison to a different daily treatment to that used in the current study. In that study, the comparator product contained a more potent corticosteroid (mometasone furoate) but had a smaller daily dose volume than the comparator in the current study. The smaller dose volume may not allow for sufficient contact between the product and the entire ear canal to rapidly reduce pruritus. Unlike the current study, Noli et al. [9] found that no difference in percentage improvement of OTIS-3 at D7 was seen between the gel and the comparator daily treatment. However, in that study, the gel provided significantly greater percentage improvements in cytology scores at Day 7 and Day 28. This suggests that the gel is capable of producing significantly faster responses to treatment than some daily treatments.

Approximately one third of dogs had recurrent otitis externa and in a little more than 10% the otitis was defined as chronic with those numbers comparatively lower than reported in one large epidemiological study [2]. This discrepancy may be explained by the fact that studies are frequently published by referral institutions and in contrast the practices involved in this study were first opinion. As in other studies, most dogs were presented with bilateral otitis, although the number of dogs with unilateral otitis externa was higher in this study than in reported studies (30% versus 6–7%) [2, 17], again probably reflecting the early presentation more typical for first opinion practice.

Staphylococci were the most common bacteria cultured from the dogs in this study, which is in accordance with other publications [17–20]. *P. aeruginosa* was cultured in a smaller number of dogs, also similar to other publications [17, 20]. In contrast with other reports [21, 22], the smaller number of dogs infected with *Pseudomonas* in this study probably reflects early presentation and first opinion practice. A large number of dogs showed *Malassezia* organisms, either with or without concurrent bacteria. These findings are in concordance with other studies [2, 3, 17], although in most publications, the exact number of dogs showing *Malassezia* organisms only versus concurrent bacterial and yeast infection was not stated.

The OTIS-3 scale used in this study was validated in a recent study [16]. A total score of ≥4 differentiated healthy from diseased ears with a specificity of 100% and a sensitivity of over 90%. Based on that publication, an OTIS-3 ≥ 5 was a criterion of inclusion in this study and an OTIS-3 of ≤3 was considered an ear in clinical remission. Utilizing this score, slightly less than three quarters of the dogs included in this study were in clinical remission after two treatments with the gel and approximately two thirds after five treatments with the suspension. This compares favorably to a number of other studies. In a study using a zinc-acetic acid ear cleaner for 14 days, 25% of the dogs with otitis externa due to *M. pachydermatis* had a clinical score indicating clinical remission [23]. In another study, 140 dogs were treated with either a marbofloxacin/dexamethasone/clotrimazole suspension daily or with miconazole/polymixin B/prednisolone daily for 7 to 14 days. The cure rate by day 14 was 58 and 41% respectively, defined by absence of erythema or ulceration, pruritus, pain and smell [24]. The latter study was also conducted in first opinion practices. A different method of scoring was used in each of these 3 studies making direct comparison difficult. One small study treating 12 dogs with twice daily ear cleaner and ticarcillin for up to 1 month had a better success rate, all but one dog responded well [25]. However, this study was not controlled, the dogs were referred for chronic otitis and treated much more aggressively including repeated procedures of ear flushing under anaesthesia. Owners used an ear cleaner twice daily and the ear medications were used for more than 2 weeks.

Cytology of the ear swabs showed a significant reduction of yeast and bacteria during treatment in both groups. On D28, approximately two thirds of the population and on D56, approximately half of the population showed no or few bacteria cytologically. Only a few studies have evaluated cytology subsequent to the treatment of otitis externa, and the methods differed in each study, thus comparisons are difficult. In a study using a

zinc-acetic acid ear cleaner for 14 days, 58% of the dogs with otitis externa due to *M. pachydermatis* had few or no yeast after 14 days [23]. In a study of dogs with *Malassezia* otitis treated with either miconazole/ dexamethasone/saline or the same solution with an added chelating agent the clinical scores and cytological scores for yeast organisms decreased significantly, but it was not stated how many dogs were negative on cytology and in remission clinically [26]. The apparent increase in bacterial cytological counts between D28 and D56 is likely to be as a result of recolonization of the ear canal by the normal flora.

Determination of MICs in this study demonstrated that most routinely identified pathogens associated with otitis externa are highly susceptible to both florfenicol and terbinafine. It was noted that despite the higher MIC, the clinical success rate in cases with *P. aeruginosa*, whilst lower than clinical success rates for *S. pseudintermedius,* was similar in both groups at D28. This is likely because topically applied antibiotics will more often than not exceed MICs established for systemic administration, even for apparently resistant organisms [27]. Although changes in MIC were observed in approximately 40% of cases at D28 compared to baseline, these were never by more than one dilution. It is considered that this is due to normal variation in the reproducibility of MIC data, as the changes were in both directions from baseline. Thus, despite the persistence of the antimicrobials within the ear canal [28], no evidence of a change in susceptibility of the target organisms was observed in this study.

Adverse events were seen in approximately 15% of the dogs in this study. However, it is important to note that adverse events were classified as any unfavorable and unintended event during or after administration of the medications, whether or not considered to be product related [29]. Adverse events by and large could be divided into two major groups. Firstly, clinical signs such as pruritus, dermatitis, conjunctivitis, otitis, or pyoderma which were most likely related to the primary cause of the otitis externa; and secondly clinical signs such as vomiting or diarrhoea, which were unlikely to be related to the underlying cause of otitis. Half of all dogs with allergic skin disease are presented with otitis externa [4, 30]. Allergic skin disease frequently waxes and wanes depending on the offending allergens and is associated with pruritus and secondary infections [4, 30], thus it is not unreasonable to assume that increased generalized pruritus in a patient is more likely due to its underlying allergy than due to the application of an otic topical. It was not the goal of the study to determine the underlying predisposing causes and as such no detailed history of other clinical signs was recorded, which would have been useful to determine whether indeed these adverse events were previously present. The second large group of adverse events included signs such as abdominal pain, pseudopregnancies, arthritis and vomiting. These clinical signs were most likely not related to the otitis externa, its primary cause or its treatment. Arthritis is a common complaint of older dogs [31] and the age of dogs included in this study was up to 16 years with a mean of 6.0 years. Gastrointestinal signs such as vomiting and diarrhea are frequently seen in dogs [32, 33], their cause is often unknown and they often resolve without specific therapy. Only three dogs (all treated with the otic gel) were withdrawn from the study due to serious adverse events, which were gastrointestinal neoplasia, obstruction due to intestinal foreign bodies and a septic abdomen 6 weeks after enrolment. These are highly unlikely to be related to the treatment of the otitis externa. Overall, adverse effects were considered not to be related to treatment, but rather to underlying or concurrent diseases and no clinically relevant changes were seen in any serum biochemistry or hematology parameter.

Conclusion

The non-inferiority of the otic gel to the otic suspension as a reference product was demonstrated following two administrations of the gel at a one-week interval. Clinical signs of the dogs with otitis externa improved similarly in the two groups, independent of the nature and chronicity of the infection. In both groups there was a significant decrease in otic scores and microorganisms as well as a rapid improvement in pain and pruritus scores. The otic gel was well tolerated in a wide range of ages and breeds of dog and thus offers a safe and effective treatment alternative for dogs with otitis externa with the added benefit of requiring only two administrations 1 week apart, providing the potential for improved compliance with the treatment regimen.

Abbreviations

ANCOVA: Analysis of covariance; CLSI: Clinical and Laboratory Standards Institute; D: Day; ITT: Intent to treat; MIC: Minimum inhibitory concentration; OTIS-3: Otitis index score; PP: Per protocol; SD: Standard deviation

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Authors' contributions

SBK and SLF were involved in the study design, in-life phase of the study, and interpretation of results and writing of the manuscript. KPD was involved in the interpretation of results and writing of the manuscript. WS was responsible for data analysis, and review of the manuscript. All authors contributed to later versions and agreed with the manuscript.

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The analgesic effects of buprenorphine (Vetergesic or Simbadol) in combination with carprofen in dogs undergoing ovariohysterectomy

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Abstract

Background: Buprenorphine is a potent lipophilic opioid analgesic that is largely used in the multimodal treatment of acute pain. Simbadol (buprenorphine hydrochloride) is the first and only FDA-approved high-concentration formulation of buprenorphine for use in cats. The aim of this study was to evaluate the analgesic efficacy of carprofen in combination with one of two commercial formulations of buprenorphine (Simbadol and Vetergesic, 1.8 mg/mL and 0.3 mg/mL, respectively) in dogs undergoing ovariohysterectomy. Twenty-four dogs were included in a randomized, prospective, controlled, clinical trial. Patients were randomly divided into 2 groups as follows. Dogs were premedicated with acepromazine (0.02 mg/kg) and either 0.02 mg/kg of Vetergesic or Simbadol intramuscularly (Vetergesic group – VG; Simbadol group – SG, respectively; $n = 12$/group). General anesthesia was induced with propofol and maintained with isoflurane in 100% oxygen. Carprofen (4.4 mg/kg SC) was administered after induction of anesthesia. Heart rate, respiratory rate, blood pressure, pulse oximetry, pain scores using the Glasgow Composite Pain Scale Short Form (CMPS-SF), sedation scores using a dynamic interactive visual analogue scale and adverse events were evaluated before and after ovariohysterectomy by an observer who was unaware of treatment administration. If CMPS-SF scores were ≥ 5/20, dogs were administered rescue analgesia (morphine 0.5 mg/kg IM). Statistical analysis was performed using linear mixed models and Fisher's exact test ($p < 0.05$).

Results: Pain and sedation scores and physiological parameters were not significantly different between treatments. Three dogs in VG (25%) and none in SG (0%) required rescue analgesia ($p = 0.109$). Adverse effects (i.e. vomiting and melena) were observed in two dogs in SG and were thought to be related to stress and/or nonsteroidal anti-inflammatory drug toxicity.

Conclusions: The administration of buprenorphine with carprofen preoperatively provided adequate postoperative analgesia for the majority of dogs undergoing OVH without serious adverse events. Prevalence of rescue analgesia was not significantly different between groups; however, it could be clinically relevant and explained by a type II error (i.e. small sample size). Future studies are necessary to determine if analgesic efficacy after Simbadol and Vetergesic is related to individual variability or pharmacokinetic differences.

Keywords: Analgesia, Buprenorphine, Canine, Ovariohysterectomy, Pain, Simbadol, Vetergesic

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Background

Ovariohysterectomy (OVH) is commonly performed in dogs and results in postoperative pain that is associated with behavioral changes [1, 2]. Safe and effective pain management is important in patient care and new analgesic techniques are constantly evolving to address this need. Buprenorphine is a potent semisynthetic highly lipophilic opioid analgesic that is largely used in the multimodal treatment of acute pain. The drug has a complex pharmacologic profile but is generally considered as a partial mu opioid agonist [3, 4]. Buprenorphine causes negligible cardiovascular effects and it is used for the treatment of mild to moderate pain such as ovariohysterectomy (OVH) in dogs and cats because of its long-lasting analgesic properties and few adverse-effects [4–6]. Indeed, a dose of 0.02 mg/kg of buprenorphine combined with a non-steroidal anti-inflammatory drug (NSAID) has been recommended for postoperative analgesia in dogs undergoing ovariohysterectomy [7, 8].

There are different commercial products of buprenorphine in the market with concentrations of 0.3 mg/mL (e.g. Vetergesic; buprenorphine hydrochloride; Champion Alstoe, Whitby, ON, Canada). On the other hand, Simbadol (buprenorphine hydrochloride, Zoetis, Parsippany, New Jersey, USA) has a concentration of 1.8 mg/mL and is the first and only FDA-approved opioid analgesic for use in cats to provide 24-h postoperative pain control after a single dose and can be administered for up to 3 days [9]. There is currently a clinical interest in using Simbadol for postoperative pain relief in dogs with the current opioid shortage in veterinary medicine in the United States [10]. In addition, it is not known whether different commercial products of buprenorphine could be used interchangeably or if drug concentrations would impact postoperative analgesia.

The aim of this study was to evaluate the analgesic efficacy of carprofen in combination with one of two commercial formulations of buprenorphine (Simbadol or Vetergesic) in dogs undergoing ovariohysterectomy. In addition, the physiological and adverse events produced by the two treatments were recorded. Our hypothesis was that the administration of Simbadol or Vetergesic with carprofen would produce similar postoperative pain scores and prevalence of rescue analgesia without serious adverse events that would require medical treatment.

Results

Age, body weight, body condition score, hematocrit, total protein, surgery and anesthesia times, and time to extubation are presented in Table 1. Surgery and anesthesia times were significantly longer in SG when compared with VG ($p = 0.032$ and $p = 0.028$, respectively).

Physiological parameters

Physiological parameters are presented in Table 2. There were no significant differences between treatment groups.

Sedation scores

There were no significant differences between treatments. In both groups, DIVAS was significantly higher 15 min after premedication, and at 0.5, 1, 2, 3, 4 and 6 h when compared with baseline values ($p < 0.001$ for all time points) (Table 2).

Pain scores

There were no significant differences between treatments. Pain scores were significantly higher at 0.5, 1 ($p < 0.001$) and 4 h ($p = 0.002$) in VG and at 0.5 h ($p = 0.002$) in SG when compared with baseline values (Table 2). Rescue analgesia was administered to three dogs in VG (3/12 dogs; 25%), and none in SG (0/12 dogs; 0%). Prevalence of rescue analgesia was not significantly different between treatment groups ($p = 0.109$). Dogs in VG that required rescue analgesia had variable body weights (21.9, 3.6 and 2.4 kg) and the same body condition score (5).

Adverse events

In SG, one dog presented vomiting and melena and another dog presented melena at 8 h postoperatively. Physical and clinical pathology examinations (complete blood count and serum biochemistry profile) were unremarkable in these two dogs. Maropitant (1 mg/kg, 10 mg/mL, Cerenia; Zoetis, Kirkland, QC, Canada by the subcutaneous route) was administered once in the dog with vomiting and melena; omeprazole (0.5 mg/kg/SID, 10 mg/tab, Losec, AstraZeneca, ON, Canada) and metronidazole (13 mg/kg/BID, 250 mg/tab, AA pharma inc., Toronto, ON, Canada) were administered for 7 days in both dogs. Adverse events were not recorded again and both dogs fully recovered within 12–24 h upon return to their shelter facilities. Total number of dogs that developed an adverse event was not significantly different between treatment groups ($p = 0.239$).

Hypothermia (defined as rectal temperature below 36.5 °C) [11] was observed in two dogs in VG and three dogs in SG at 0.5 h, in one dog in VG and two dogs in SG at 1 h, and in one dog in each group at 2 h. Prevalence of hypothermia was not significantly different between groups (times 0.5 and 1 h: $p = 0.5$; time 2 h: $p = 0.761$).

Discussion

This study demonstrated that the intramuscular administration of Simbadol provides effective postoperative analgesia in combination with carprofen in dogs undergoing

Table 1 Demographic data, surgery and anesthesia time, and time to extubation of dogs undergoing ovariohysterectomy

Variable	Vetergesic ($n = 12$)	Simbadol (n = 12)	p value
Body weight (kg)	12.1 (9.7)	19.3 (10.9)	0.219
Body condition score (1–9)	5 (4–5)	5 (4–6)	0.378
Age (years)	2.7 (2)	4.2 (3)	0.319
Hematocrit (%)	40.4 (4.5)	41.6 (2.3)	0.378
Total protein (g/dL)	6.3 (0.75)	6.7 (0.79)	0.216
Surgery time (min)	34.8 (8.9)	44.9 (12.4)	0.032
Anesthesia time (min)	47.8 (8.4)	56.7 (12)	0.028
Time to extubation (min)	8.3 (3.4)	8.5 (2.9)	0.849

Dogs treated with carprofen in combination with two concentrations of buprenorphine (Vetergesic or Simbadol). Values are expressed as mean (SD) with the exception of body condition score which is reported as median (range)

OVH. Pain scores and prevalence of rescue analgesia were not significantly different between SG and VG; however, one may argue that differences in rescue analgesia could be of clinical relevance. The observed analgesic effect was different between treatments (prevalence of rescue analgesia: 25% of dogs in VG versus 0% dogs in SG), albeit not statistically significant. Had one more dog been administered rescue analgesia in VG, the difference would have been significant between treatments. Indeed, the lack of difference between groups could be related to a type II error due to small sample size suggesting a clinically relevant finding masked by potential low statistical power. On the other hand, this finding is difficult to explain because the only difference between these two commercial products is the concentration of buprenorphine (1.8 and 0.3 mg/mL for SG and VG, respectively) which results in different final volumes of injection for the same dose. The rate of diffusion of a compound across a membrane depends on its concentration gradient across membranes, liquid/partition coefficient and diffusion coefficient of the drug (Fick's law of diffusion) [12]. In this study, the rate of diffusion should be similar for Simbadol and Vetergesic because the same active ingredient, dose, route of administration and body location for injection were used; excipients are similar between these two drugs. Contrarily, a study in cats showed that the concentration of buprenorphine (0.3, 0.6 and 1.2 mg/mL) influenced maximum plasma concentrations but not time to peak effect or thermal antinociception [13]. If the same findings were corroborated in this study, higher plasma concentrations of buprenorphine in SG would allow the drug to transfer down the concentration gradient into the central nervous system allowing the drug to occupy more opioid receptors and to produce greater analgesic effect [14]. A pharmacokinetic study is necessary to determine whether these differences are a) due to absorption, distribution, metabolism or elimination, or if b) perhaps these three dogs in VG had lower nociceptive thresholds for pain or c) buprenorphine was not the best opioid choice for these individuals. In any of these scenarios, rescue analgesia would have been administered even if these three dogs had been allocated to SG instead of VG. Finally, dogs in SG were heavier than in VG. It is possible that dogs in VG could have received a smaller dose than SG if doses had been based on body surface area. In this case, treatments would not have been equivalent. However, labelled dose recommendations for buprenorphine do not take in consideration body surface area in veterinary medicine.

Pain scores were also not significantly different between VG and SG. This result is not surprising because scores were excluded from statistical analysis following administration of rescue analgesia. This approach may overestimate the analgesic effect of a treatment because higher pain scores are possibly omitted, and selection bias is introduced while avoiding analysis bias. This approach limits the ability to detect significant differences among treatments using pain scores; however, this should not be an issue when prevalence of rescue analgesia is used as the main outcome of a clinical trial [15].

Surgery and anesthesia time were significantly longer in SG when compared with VG most likely due to differences in mean body weight. Dogs in SG were heavier than VG even if not significantly different and OVH would naturally take more time in the first than the latter group. Even so, surgery and anesthesia times were shorter in this study than previous similar reports [7, 8].

Some physiological parameters including HR, SAP, MAP and DAP were significantly lower after premedication than baseline values. Acepromazine produces peripheral vasodilation and reduces blood pressure due to antagonism of α1- adrenergic receptors. Decreases in HR and blood pressure may be observed after the administration of buprenorphine due to increased vagal stimulation [6]. Sedation and decreases in catecholamine concentrations via dopaminergic effect may result in lower HR after premedication and these results are not surprising. Physiological values in the current study were within normal ranges and these changes were not clinically relevant [4, 16]. Both concentrations of buprenorphine did not induce important cardiorespiratory

Table 2 Pain and sedation scores and physiological parameters in dogs undergoing ovariohysterectomy

Time points	Drugs	CMPS-SF	DIVAS	Temperature (°C)	HR (bpm)	RR (bpm)	SAP (mmHg)	MAP (mmHg)	DAP (mmHg)	SpO2 (%)
Time 0 (baseline values)	VG	0 (0)	0 (0)	38.5 (38.2–38.8)	125 (107–143)	37 (30–44)	159 (141–177)	116 (104–127)	91 (79–102)	97 (96–98)
	SG	0 (0)	0 (0)	38.5 (37.9–39.0)	117 (105–129)	37 (30–44)	167 (145–188)	123 (111–134)	100 (89–110)	96 (95–98)
15 min after premedication	VG		22.3 (18.4–26.3)	38.2 (38.1–38.3)[a]	94 (80–108)[a]	34 (28–40)	146 (125–166)	100 (89–111)[a]	75 (67–84[a]	98 (97–99)
	SG		23.7 (13.4–33.9)	38.1 (37.9–38.3)[a]	100 (86–115)	34 (27–40)	140 (133–146)[a]	97 (90–104)[a]	77 (70–83)[a]	96 (95–97)
Postoperative 0.5 h	VG	1.25 (0.6–1.9)	38.9 (28.1–49.7)	37.0 (36.7–37.4)[a]	102 (84–120)[a]	34 (30–38)	157 (143–172)	117 (104–130)	95 (84–106)	97 (96–97)
	SG	0.92 (0.4–1.4)	38.1 (24.2–52)	36.8 (36.5–37.1)[a]	109 (98–121)	29 (24–34)	161 (153–170)	118 (113–124)	95 (89–101)	97 (96–97)
Postoperative 1 h	VG	1.5 (0.4–2.6)	27.8 (16,5–39)	37.1 (36.8–37.5)[a]	94 (76–112)[a]	31 (25–37)	158 (145–172)	114 (102–126)	87 (79–96)	97 (96–98)
	SG	1 (0.6–1.4)	32.7 (19.9–45.5)	37 (36.7–37.3)[a]	105 (94–116)	31 (27–36)	153 (141–164)[a]	115 (109–122)	93 (87–99)	96 (95–97)
Postoperative 2 h	VG	1.27 (−0.1–2.7)	18.9 (15.5–22.4)	37.2 (36.9–37.6)[a]	91(75-108)[a]	29 (24–35)	151 (136–165)	105 (95–115)	83 (74–92)	97 (96–99)
	SG	0.5 (0.1–0.9)	22 (16.3–27.4)	37.2 (36.9–37.5)[a]	103 (89–118)	31 (27–35)	161 (153–169)	115 (109–121)	90 (82–99)	96 (95–97)
Postoperative 3 h	VG	1 (−0.3–2.3)	13.9 (11.3–16.5)	37.4 (37.1–37.6)[a]	92 (68–116)[a]	31 (26–35)	157 (143–170)	108 (100–116)	82 (76–88)	97 (96–98)
	SG	0.7 (0.3–1.1)	15.3 (11.2–19.3)	37.4 (37.3–37.6)	88 (79–97)[a]	32 (27–36)	156 (147–166)	113 (108–119)	89 (84–95)	97 (96–98)
Postoperative 4 h	VG	0.9 (0.3–1.5)	10.4 (6.5–14.4)	37.4 (37.2–37.6)[a]	86 (66–105)[a]	32 (27–37)	157 (139–176)	116 (105–127)	90 (82–98)	97 (96–99)
	SG	0.5 (0.1–0.9)	12.3 (7.9–16.6)	37.5 (37.4–37.7)[a]	88 (77–99)[a]	32 (27–36)	159 (149–169)	110 (104–116)[a]	85 (79–90)[a]	96 (95–97)
Postoperative 6 h	VG	0.3 (−0.4–1.1)	6.8 (3.8–9.8)	37.6 (37.4–37.8)[a]	92 (74–109)[a]	31 (24–39)	155 (138–172)	109 (99–119)	82 (73–91)	97 (96–98)
	SG	0.2 (−0.1–0.4)	6.8 (4.4–9.1)	37.6 (37.4–37.9)[a]	96 (85–107)[a]	33 (28–37)	160 (148–171)	114 (106–122)	90 (84–96)	97 (96–97)
Postoperative 8 h	VG	0.1 (−0.2–0.4)	4.1 (0.8–7.3)	37.8 (37.5–38.0)[a]	89 (78–100)[a]	31 (26–37)	158 (139–178)	107 (96–118)	82 (73–91)	97 (96–99)
	SG	0.1 (−0.1–0.3)	3.3 (2.1–4.4)	37.9 (37.7–38.0)[a]	101 (87–114)	33 (29–38)	159 (146–172)	110 (110–120)[a]	88 (79–96)[a]	97 (96–98)

Mean (CI) for pain scores using the Glasgow Composite Pain Scale short-form (CMPS-SF), sedation scores using the dynamic and interactive visual analogue scale (DIVAS) and physiological parameters including temperature, heart rate (HR), respiratory rate (RR), systolic (SAP), mean (MAP) and diastolic (DAP) blood pressure, and pulse oximetry (SpO_2) in dogs undergoing ovariohysterectomy and treated with carprofen in combination with two concentrations of buprenorphine (Vetergesic - VG or Simbadol - SG)

[a]Significant difference when compared with baseline values

changes in dogs undergoing OVH. Hypothermia was observed in some dogs in both groups up to 2 h postoperatively. Decreases in temperature are commonly observed after general anesthesia and its prevalence was not statistically different between groups.

Vomiting and/or melena were observed in two dogs in SG at 8 h postoperatively. Main differential diagnosis for these gastrointestinal clinical signs include stress, primary gastrointestinal disease and NSAID toxicity.

Gastrointestinal toxicity is recognized as one of the most common signs of NSAID toxicity and they could be related to individual sensitivity to NSAID administration or an idiopathic reaction [17]. However, carprofen has been administered in several studies for acute pain management without clinically relevant adverse events [17]. It may be also possible that dogs did not have enough time to acclimate to the hospital setting after transportation presenting with stress-induced gastrointestinal disorder. To the authors' knowledge, these clinical adverse effects have not been reported after the administration of buprenorphine in dogs and were not considered to be treatment- (Vetergesic or Simbadol) related.

Conclusion

The administration of carprofen with either Simbadol or Vetergesic preoperatively provided adequate postoperative analgesia for the majority of dogs undergoing OVH and without serious adverse events. Prevalence of rescue analgesia was not significantly different between groups; however, it could be clinically relevant and explained by a type II error (i.e. small sample size). Future studies are necessary to determine if analgesic efficacy after Simbadol and Vetergesic is related to individual variability or pharmacokinetic differences.

Methods

This study was a prospective, randomized, blinded, controlled, clinical trial conducted at Université de Montréal. The study was approved by the animal care committee of the Université de Montréal (16-Rech-1846). This study follows The Consolidated Standards of Reporting Trials (CONSORT) [18].

Animals

Twenty-four adult female dogs from shelter facilities were enrolled to undergo OVH. Dogs were included if they were considered healthy based on medical history, complete physical examination and hematocrit and total protein. Dogs had to be up to date on vaccination and parasite control. Exclusion criteria included aggression, anxiety, pregnancy or any sign of disease. Dogs were admitted approximately 16 h before surgery. Food but not water was withheld for 8–12 h.

Anesthetic protocol, surgery and treatments

Dogs were randomly allocated in one of two groups (Vetergesic group – VG or Simbadol group – SG) ($n = 12$/group). Randomization was performed by an individual not involved in pain assessment using a random permutation generator (www.randomization.com). Premedication was performed with acepromazine (0.02 mg/kg; Acepromazine maleate, Gentès & Bolduc, Saint-Hyacinthe, QC, Canada) and either 0.02 mg/kg of Vetergesic (VG) or Simbadol (SG) by the intramuscular route of administration (i.e. epaxial muscles). Approximately 20 min later, an intravenous catheter was aseptically introduced in a cephalic vein and induction of anesthesia was performed with intravenous administration of propofol (10 mg/mL, Propoflo 28, Zoetis, Kirkland, QC, Canada) to effect. After intubation with an appropriately sized endotracheal cuffed tube, dogs were maintained with isoflurane (Isoflurane USP, Fresenius Kabi, Toronto, ON, Canada) in 100% oxygen, and received carprofen (4.4 mg/kg; 50 mg/mL, Rimadyl, Zoetis, Kirkland, QC, Canada) by the subcutaneous route approximately five minutes after anesthetic induction. Anesthetic monitoring was performed according to previously published guidelines [19]. OVH was performed by the same veterinarian with previous experience in surgery. A ventral midline incision was made through the skin, subcutaneous tissue and the aponeurosis of the rectus abdominis muscle and a modified 2-clamp technique was employed. The abdominal wall and subcutaneous tissues were closed using simple continuous pattern of absorbable sutures. The skin was closed using simple interrupted pattern of non-absorbable suture. Surgery time (time elapsed from the first incision until placement of the last suture), anesthesia time (time elapsed from induction of propofol to turning off the vaporizer dial) and time to extubation (time elapsed from turning off the vaporizer dial until extubation) were recorded.

Data collection

Evaluations were performed before premedication which was approximately 60 mins prior to the induction of anesthesia (time 0, baseline), 15 min after premedication and at 0.5, 1, 2, 3, 4, 6 and 8 h after the end of surgery by an observer who was unaware of treatment administration.

Physiological parameters

Temperature, heart rate (HR), respiratory rate (RR), systolic (SAP), mean (MAP) and diastolic (DAP) blood pressure and pulse oximetry (SpO_2) were recorded. Temperature was measured using a rectal thermometer. HR and RR were recorded via thoracic auscultation. SAP, MAP and DAP were obtained via a non-invasive oscillometric blood pressure device (petMAP, Ramsey Medical Inc., Tampa, FL, USA). The cuff was positioned proximal to the carpus and cuff size was chosen according to the manufacture's direction. Blood pressure was measured at a level of right atrium three times at each time point and average values were used [20]. SpO_2 was measured using a pulse oximeter (Rad-5 V, Masimo, Irvine, CA, USA). The probe was placed on the skin between the digits of limbs or over the ears.

Sedation scores

Sedation scores were evaluated using the dynamic and interactive visual analogue scale (DIVAS) where 0 was considered as no sedation and 100 as maximum sedation at the aforementioned time-points [21].

Pain scores

The Glasgow Composite Pain Scale short-form (CMPS-SF) was used to evaluate pain at the aforementioned time points with the exception of 15 min after premedication. The CMPS-SF is a validated instrument for use in measuring acute pain in dogs [22, 23]. It includes 30 descriptor options within six behavioral categories. Within each category, the descriptors are ranked numerically according to their associated pain. The

maximum pain score is achieved with 24 points. For this study, lameness scores (section B of the CMPS-SF) were not included in the evaluation since some dogs could not ambulate due to residual effects of anesthesia. Therefore, rescue analgesia (morphine 0.5 mg/kg, 10 mg/mL, Morphine Sulfate Injection, Sandoz Canada Inc., Boucherville, OC, Canada via intramuscular route of administration) was provided if CMPS-SF scores were $\geq 5/20$. For scoring, the dogs were initially evaluated inside their cages without being disturbed. Pain and sedation scores and physiological data were discarded after rescue analgesia and not included in the statistical analysis to avoid bias. However, all dogs were evaluated until the end of the study.

Adverse events

Adverse event was defined as any undesirable experience/observation (expected or not) that occurred after administration of the test items whether considered or not to be related to the product [24].

Statistical analyses

Statistical analyses were performed using standard statistical software (SPSS Statistics V25, IBM, Armonk, NY, USA). Power analysis was calculated before the study and indicated that a minimum sample size of 8 dogs per group would be needed to detect a difference of 3 points between the 2-means using CMPS-SF and considering an alpha value of 0.05, a power of 80% and a standard deviation within group of 2 points [25]. Data were tested for normality using a Shapiro-Wilk test. Demographic data for each treatment group were compared using independent t-test or Mann-Whitney U test where appropriate. All physiological parameters, DIVAS sedation and CMPS-SF were compared between treatments and time points using a linear mixed model for repeated measures. Time point and treatment group, and their interaction were considered as fixed effects. Dog was considered a random effect and body weight was added as a covariate to the model. The best structure of the covariance was assessed using information criteria that measured the relative fit of a competing covariance model. The Benjamini–Hochberg procedure was used to adjust for multiple comparisons. Total number of rescue analgesia and prevalence of adverse events were compared between treatment groups using Fisher's exact test. Values of $p < 0.05$ were considered statistically significant.

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Authors' contributions

RW collected and analyzed data and drafted the first version of the manuscript. BM participated in the development of methodology, performed surgeries and statistical analysis. ME performed general anesthesia and technical assistance. AC was responsible for patient recruitment, admission and discharge and technical assistance. DE participated in the conceptualization, funding acquisition and development of methodology. PS participated in the conceptualization, funding acquisition, development of methodology, manuscript preparation and was the principal investigator of the study. All authors read and approved the final manuscript.

Consent for publication

Not applicable.

Competing interests

Dr. Paulo Steagall has received speaker honoraria and provided consultancy services to Zoetis. Dr. Beatriz Monteiro has provided consultancy services for Zoetis. Dr. Daniel Edge is an employee of Zoetis. This does not alter the authors' adherence to BMC Veterinary Research policies on sharing data and materials.

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The contributors of this book come from diverse backgrounds, making this book a truly international effort. This book will bring forth new frontiers with its revolutionizing research information and detailed analysis of the nascent developments around the world.

We would like to thank all the contributing authors for lending their expertise to make the book truly unique. They have played a crucial role in the development of this book. Without their invaluable contributions this book wouldn't have been possible. They have made vital efforts to compile up to date information on the varied aspects of this subject to make this book a valuable addition to the collection of many professionals and students.

This book was conceptualized with the vision of imparting up-to-date information and advanced data in this field. To ensure the same, a matchless editorial board was set up. Every individual on the board went through rigorous rounds of assessment to prove their worth. After which they invested a large part of their time researching and compiling the most relevant data for our readers.

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The publishing team has been an ardent support to the editorial, designing and production team. Their endless efforts to recruit the best for this project, has resulted in the accomplishment of this book. They are a veteran in the field of academics and their pool of knowledge is as vast as their experience in printing. Their expertise and guidance has proved useful at every step. Their uncompromising quality standards have made this book an exceptional effort. Their encouragement from time to time has been an inspiration for everyone.

The publisher and the editorial board hope that this book will prove to be a valuable piece of knowledge for researchers, students, practitioners and scholars across the globe.

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Veterinary Medicine: Assessment, Diagnosis and Treatment of Animal Diseases

About the Book

Veterinary medicine is the discipline that focuses on the prevention, diagnosis and treatment of animal disorders, injuries and diseases. It deals with all animal species, both domestic and wild. Professionals in this field are known as vets or veterinary surgeons. They might be assisted by veterinary technicians and nurses. The diagnosis of diseases in animals is primarily based on clinical signs. This is due to the fact that animals are unable to vocalize their symptoms. In certain cases, the results of diagnostic tests such as radiography, MRI and CT scans, are also consulted. Some of the specialties within veterinary medicine are anaesthesiology, immunology, oncology and radiology. This book explores all the important aspects of veterinary medicine in the present day scenario. Some of the diverse topics covered in this book address the varied branches that fall under this field. It will help new researchers by foregrounding their knowledge in veterinary medicine.

About the Editor

Kevin McLeod pursued his MSc in Veterinary Sciences from University of Bristol, United Kingdom. He is a guest lecturer and teaches courses on different zoonotic diseases such as rabies, influenza and cutaneous anthrax infection as well as their diagnosis and treatment. McLeod has spent more than 8 years in the veterinary medicine industry, and has authored numerous articles and books, which have been published worldwide. He has been an eminent speaker in seminars across Europe, United States, Canada and Middle Asia.

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