

# Embryo Transfer in Sheep and Goats

## A training manual

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## Introduction

Embryo transfer (ET) is a method of assisted reproduction based on producing multiple embryos in a female donor (genetically superior mother) that are then transferred to various female recipients (gestating mothers).

Hormonal treatments to induce multiple ovulation and ET enable the intensive use of genetically superior females. Over the last 25 years, considerable progress in ET has been achieved, improving the genetic quality of sheep and goats. This has required maximizing embryo production and survival to obtain offspring of high genetic value. It must be emphasized that a genetically superior mother can be used in ET programs more than once, thus multiplying her reproductive potential by using genetically inferior animals as recipients of genetically superior embryos (Mueller 1993).

The first embryo transfers in sheep and goats were carried out 75 years ago (Warwick *et al.* 1934). After the 1960s, they were continued in Australia (Moore and Rowson 1960) and in New Zealand (Tervit and Havick 1976), which helped to clarify the conditions and possibilities of this biotechnology.

The natural reproductive potential of each species and breed is a limiting factor for the diffusion of genetic improvements. Under traditional sheep and goat breeding conditions, the number of offspring from a female per year is one or two. Therefore, during the female reproductive lifetime, six to eight offspring can be obtained. Embryo transfer increases the reproductive potential of high genetic value females by taking advantage of the great oocyte reserve in the ovary. Hormonal stimulation of the ovaries induces multiple ovulations, resulting in an increase in the average ovulation rate of a given breed. Consequently, a considerable number of offspring can be obtained in a short time.

Embryo transfer shortens the generational interval, resulting in greater genetic progress. At the same time, artificial insemination and ET together constitute excellent tools for genetically improving flocks and herds isolated from suppliers of male improvers.

Over the last 20 years, embryo transfer has been used in Australia and New Zealand to incorporate Angora goat genetic material with little health risk. In France, a program for genetically improving dairy sheep is underway (the Lacaune breed, used in the manufacture of Roquefort cheese). International trade in frozen sheep and goat embryos has led to the world-wide diffusion of germplasm, with very low health risks. As a consequence, there have been rapid genetic improvements in different breeds and the establishment of alternate meat, milk, wool and hair production (e.g. mohair or cashmere) choices.

This technique is used in domestic animals, fundamentally for genetic, commercial, health and species conservation reasons. The following objectives are pursued:

- Introduction and rapid diffusion of new breeds or genotypes of high productive value. Desired characteristics are rapidly introduced into flocks or herds in only one generation. For example, the genetic characteristics of the Merino 'prolific Booroola gene' that has given added value to carriers and which can be rapidly multiplied through ET.
- Reduction in the risk of disease transmission because, in the initial stages of embryo development, there is natural protection from infective agents.
- Diffusion of genetic material of high commercial value and with easy environmental adaptation of offspring to different production and management systems.
- Increased genetic progress in production. This results from intensified selection of mothers destined for production of superior males, as a consequence of there being more numerous offspring per selected female.
- Shortened generational interval. Being able to obtain offspring from younger mothers shortens the interval between generations. Further improvement follows if semen from young males is used.
- Provision of support for reproductive techniques involving micromanipulation of embryos (sex determination, in vitro fertilization, cloning, transgenesis, etc).
- Breed or species conservation. Conserving genetic material (frozen embryos) in germplasm banks ensures the conservation of genes which would otherwise be lost, leading to species disappearing.

### Principles and General Considerations of Embryo Transfer

Female donors must be selected based on their genetic value and on criteria to improve the productivity of each breed.

General factors such as reproductive conditions, health and nutritional aspects must be taken into account for both donors and recipients. Clinical examination of animals and serological tests for infectious diseases (such as brucellosis and foot and mouth disease), must be carried out.

Females must have already had at least one offspring and there must have been at least two post-birth months before hormonal treatment begins. Less time may result in fewer embryos being produced.

The need to use young females as donors may lead to lower reproductive efficiency. If it is necessary to use a female that has never given birth, the minimum weight must be 75% of the adult weight of the same breed. Furthermore, it must have already experienced estrus.

The use of yearling animals as recipient mothers is not recommended. Adult females are the best option as they can undergo gestation without compromising their own growth and also contribute to the development of offspring through lactation.

Embryo transfer implies a series of procedures in donors and recipients. When these are performed under management conditions different from the usual

ones, it is advisable to give the animals a one to two month period of adaptation before beginning treatment.

Identification by ear-tags with clearly visible numbers will facilitate intensive procedures without errors being made and without causing unnecessary stress to the animals, which could negatively affect the results.

Health, nutritional and reproductive aspects of males and semen quality must be considered, whether for natural service or artificial insemination with fresh, cooled/chilled or frozen semen.

Before undertaking an ET program, the following must be considered:

1. Hormonal physiology of reproduction.
2. Ovarian stimulation to promote multiple ovulations.
3. Factors involved in the response to multiple ovulations.
4. Induced ovulation in recipients and estrus synchronization between donor and recipient.
5. Donor fertilization.
6. Embryo recovery.
7. Identification of embryos.
8. Assessment of embryo quality.
9. Embryo transfer.
10. Embryo preservation.

### **1. Hormonal physiology of reproduction**

During ET, specific hormonal treatments are used to induce estrus and multiple ovulation (female donors), to induce ovulation (female recipients) and to synchronize estrus between both donors and recipients.

Gonadotropin-releasing hormone ( GnRH) is a decapeptide produced by secretor neurons within the central nervous system. Secretion of this hormone is influenced by external (photoperiod, stress, nutrition) and internal factors (hormones such as estrogen and progesterone, pheromones). Its action is exerted on the gonadotropic cells of the hypophysis, stimulating the synthesis and liberation of follicle stimulating hormone (FSH) and luteinizing hormone (LH).

The gonadotropins FSH and LH are glycoproteins synthesized at the level of the anterior hypophysis and they participate in the regulation of ovarian function.

FSH promotes growth, and follicle and oocyte maturation. It induces mature follicles to present LH receptors and to maintain estrogen liberation. Basal secretion is associated with follicle dynamics during the luteal phase, and presents two increases during the follicular phase: the first, together with the pre-ovulatory LH peak - which is GnRH dependent - and the other, less intense, 18 hours later and produced by a decrease in estrogen blood levels - which is not GnRH dependent.

LH concentration increases in pulses for a short time before ovulation. LH pulse frequency is subject to the stimulation of hypophysis cells by GnRH. Each LH pulse corresponds to a GnRH pulse. During the pre-ovulatory phase, the increase in estrogen secretion by the follicles exerts a positive feedback on the hypothalamus-hypophysis axis which, in turn, induces the so-called 'LH pre-ovulatory peak'.

LH participates together with FSH in the final maturation of follicles, in the liberation of oocytes (ovulation) and in the formation of the corpus luteum that develops from the follicle following the release of the oocyte.

Progesterone is a steroid secreted by the corpus luteum. If fertilization occurs, its level during gestation, derived from the placenta and corpus luteum, remains constant. Its function is to maintain pregnancy until birth. Before ovulation, together with estrogens, it plays a role in the external manifestations of estrus. It has a negative feedback on the hypothalamus, inhibiting GnRH secretion and, consequently, LH pulsation, thus blocking ovulation.

Prostaglandin is secreted by the uterine endometrium. Its increase induces luteolysis (elimination of the corpus luteum) and, therefore, a decline in progesterone levels leading to the manifestation of a new estrus period.

## 2. Ovary stimulation for multiple ovulation

Multiple ovulations have been induced in sheep and goats by administering 1000 to 2000 IU of pregnant mare serum gonadotropin (PMSG) or equine chorionic gonadotropin (eCG), 48 hours before ending progestagen treatment. Treatment with PMSG has given results inferior to those obtained with FSH. Because of its high molecular weight, PMSG has a long half-life (21 hours), results in disperse follicle growth, and induces the formation of anovulatory follicles and premature follicle luteinization, reducing fertility, recovery of embryos and embryo quality (Armstrong and Evans 1983; Armstrong *et al.* 1983; Moor *et al.* 1985; Tsunoda and Sugie 1989; Walker *et al.* 1989). Multiple ovulations appear 54 hours after withdrawing progestagen intravaginal sponges (Walker *et al.* 1986).

FSH of porcine or ovine origin is more efficient (Alberio *et al.* 1993). In comparison to PMSG, FSH administration produces better sperm migration (Evans and Armstrong 1984), better fertilization rates when using artificial insemination (Evans *et al.* 1984) and higher embryo production (Armstrong *et al.* 1983; Torres *et al.* 1987).

The most widely accepted treatment for inducing multiple ovulation in sheep is by the application, in decreasing doses, of FSH towards the end of progestagen treatment. Unlike PMSG, the biological half-life of FSH is short (3 to 4 hours) and requires the administration of 6-8 applications every 12 hours. Multiple ovulations appear 60 hours after intravaginal sponge withdrawal (Photo 1) (Walker *et al.* 1986).

FSH dosing is expressed in mg Armour, which is a unit of activity in biological tests equivalent to 10 to 14 µg of pure FSH hormone. Doses recommended for sheep and goats vary between 16 and 20 mg Armour (Tervit *et al.* 1984; González *et al.* 1991; Brebion *et al.* 1992). Ovulatory response to these doses depends on genotype. An increase in the FSH dose (more than 16–20 mg Armour) produces no increase in ovulatory response. FSH doses can also be expressed in NIH-FSH-P1, total doses of up to 200 mg per female donor being recommended.

To obtain a better ovulatory response, it is advisable to increase the administration of LH towards the end of the progestagen treatment (Cognié *et al.* 1986; Baril *et al.* 1989). The recommended ratio of FSH:LH is 3:1 (first to third application), 1:1 (fourth application) and 1:2 (fifth application).

The time of the appearance of estrus after FSH treatment varies. It can usually be detected 24 to 36 hours after removal of the pessary.

'Traditional treatment' in sheep combines decreasing FSH doses with a single application of PMSG towards the end of the progestagen treatment. It consists of inserting intravaginal sponges containing progestagens (60 mg of medroxyprogesterone acetate (MAP), Syntex, Argentina) for 14 days and administering 200 mg of NIH-FSH-P1 (Folltropin-V, Bioniche, Canada) per treated ewe, applied in six decreasing doses every 12 hours as follows: 50, 50, 30, 30, 20 and 20 mg of FSH, starting on the morning of day 12 after inserting the intravaginal sponge. A single dose of PMSG (200 IU, Novormon 5000, Syntex, Argentina) is administered together with the fifth application of FSH and the progestagen removal.

Treatment in goats is similar to that in sheep. To obtain a better fertilization rate, a short treatment with progestagen by intravaginal sponge for 11 days and a dose of prostaglandin F2 alpha (50 µg cloprostenol) given 48 hours before sponge removal (Corteel *et al.* 1988), is recommended.

However, in Merino ewes and during the breeding season, we have shown that it is possible to reduce the total dose per ewe donor to 80 mg of NIH-FSH-P1, applied in six applications every 12 hours of 18, 18, 14, 14, 8 and 8 mg starting on the morning of day 12 after sponge insertion. The fifth application coincides with the withdrawal of the intravaginal sponge together with a 200 IU dose of PMSG (Novormon 5000, Syntex, Argentina) (Figure 1). In this way, although there is a lower ovulation rate ( $P < 0.05$ ) due to the lower dose, a similar number of embryos of similar quality are obtained. This is probably because of a greater embryo recovery rate and a higher fertilization rate ( $P > 0.05$ ) (Table 1). With this treatment, more than 80% of sheep donors show estrus 36 hours following pessary removal. Reducing the high cost of FSH to nearly one third implies an economic benefit when used in commercial ET programs.

It must be emphasized that it will always be necessary to determine a recommendable dose depending on the efficiency of the multiple ovulation treatment, and to adjust it based on species, breed, time of year, production system, etc.

**Table 1. Embryo yields in Merino ewes following superovulatory treatment with 80 mg (low dose) or 200 mg (high dose) of FSH (Folltropin-V) + 200 IU of PMSG (Novormon 5000) during the breeding season.**

	Low dose	High dose
Animals (n)	43	11
Ovulation rate ( $\bar{x}$ )	13.0 $\pm$ 0.9 a	17.5 $\pm$ 1.8 b
Recovered ovarian structures* ( $\bar{x}$ )	7.4 $\pm$ 0.7 a	10.0 $\pm$ 1.4 a
Ovarian structures recovery rate (%)	59.6 $\pm$ 3.6 a	56.3 $\pm$ 7.1 a
Recovered embryos ( $\bar{x}$ )	5.9 $\pm$ 0.6 a	6.4 $\pm$ 1.2 a
Embryo recovery rate (%)	50.0 $\pm$ 4.0 a	38.6 $\pm$ 7.9 a
Recovered grade 1-2 embryos** ( $\bar{x}$ )	5.0 $\pm$ 0.6 a	5.5 $\pm$ 1.2 a
Grade 1-2 embryo recovery rate (%)	85.0 $\pm$ 3.8 a	82.6 $\pm$ 7.2 a
Unfertilized oocytes ( $\bar{x}$ )	1.0 $\pm$ 0.5 a	2.6 $\pm$ 1.0 a
Non-fertilization rate (%)	9.7 $\pm$ 4.3 a	25.1 $\pm$ 8.4 a
Response rate (>3 CL) (%)	98.0 a	100.0 a

a, b indicate significant differences between treatments ( $P < 0.05$ )

\* Embryos + oocytes + zonae pellucidae \*\* See Annex 1. Grades of embryo quality (IETS 1998)

Ovulatory response to repeated multiple ovulation treatments depends on FSH source. Baril *et al.* (1992) have shown that using porcine FSH is less effective than ovine or caprine FSH. This is attributed to the development of heterospecific anti-gonadotropin antibodies. Serum can be administered, but is costly.

In goats, repeated porcine FSH treatments have caused anti-FSH antibodies (Remy *et al.* 1991) and a decrease in ovulatory response (40 to 50% in the third treatment, up to 70 to 80% in the fourth or fifth treatment).

### 3. Factors involved in the response to multiple ovulation

The intrinsic factor of each animal plays a primary role in their response to multiple ovulation treatment. Studies done in bovines showed that only 68% of females produced transferable embryos. The remaining 32% were made up of females that did not respond to ovarian stimulation, those in which embryo or oocyte recovery was not possible and those that produced non-transferable embryos (Donaldson 1984). It must therefore always be remembered that a percentage of females will not respond to multiple ovulation treatment. Individual variability in hormonal response to multiple ovulation is conditioned by extrinsic factors (breed, breeding season, nutrition) as well as intrinsic ones (folliculogenesis).

Breed constitutes an important factor of variation. The most prolific breeds show a greater response to multiple ovulation, yielding more transferable embryos and also offspring (Tervit 1986; Ritar *et al.* 1988; Baril *et al.* 1989).



Breeding season also affects the mean number of ovulations per female in sheep, being higher during the reproductive period than during the an-estrous period (Torrès *et al.* 1984). This difference was not observed in dairy goats (Baril and Vallet 1990), although embryo quality was superior during the breeding season. For the Merino breed, we have found similar ovulation rates during both the breeding and non-breeding seasons; however, a higher fertilization rate and a higher number of recovered embryos were observed during the breeding season.

Nutrition plays a fundamental role in the response to multiple ovulation treatment. It has been shown that it affects not only the ovulatory response but it may also promote premature luteolysis, both in and out of the sexual season (Baril *et al.* 1989; Jabbour *et al.* 1991). Therefore, embryo recovery is low (Armstrong *et al.* 1982; Tervit 1986). In goats, premature luteolysis is very variable (0 to 27%). This event has also been reported in sheep (Trounson and Moore 1974; Jabbour *et al.* 1991).

Ovulatory response to hormonal treatment has been studied on the basis of follicle presence in the ovaries. It has been shown that this is positively correlated to the number of small follicles (2 to 3 mm diameter) when applying the first FSH dose, to the number of medium follicles (4-5 mm) at the end of progestagen treatment and to the number of large follicles (>6 mm) at the onset of estrus (González-Bulnes *et al.* 2000). However, in Merino ewes, we found no correlation between the number of corpora lutea and the number of small, medium and large follicles, or their total number, when the superovulatory treatment was initiated.

It has been suggested that gonadotropic blocking by prolonged treatment with a GnRH antagonist (10-14 days) prior to FSH application, can increase the number of ovulations and concentrate them, and also reduce variability among animals and double the number of offspring per donor (7 lambs per donor ewe), with high repeatability (Cognié 1999; Cognié *et al.* 2003). Furthermore, intravenous administration of 3 mg of LH at between 32 to 36 hours after progestagen treatment is over, permits synchronized ovulation 20-28 hours after application and makes possible timed artificial insemination 48-50 hours after the end of progestagen treatment (Cognié *et al.* 2003). In France, this treatment has given promising results, leading to a greater number of multi-ovulated ewes (>5 ovulations), with more than 10 transferable embryos in Lacaune breed donors. However, a high ovulatory response (>30 corpora lutea per female) has been associated with a lower fertilization rate and lower number of transferable embryos (Cognié *et al.* 2003) (Table 2).

Although great individual repeatability in ovulation rate between two successive multiple ovulation treatments has been obtained with this treatment, there still persists a high variability among individuals.

**Table 2. Embryo recovery, fertilization rate and percentage of transferable embryos according to multiple ovulation rate in Lacaune breed ewes.**

Corpora lutea	Donor ewes	Recovered eggs (%)	Fertilized eggs (%)	Transferable embryos (%)
5-9	26	60	84 b	69 a
10-14	39	62	93 a	76 a
15-19	41	62	89 a	79 a
20-24	17	67	85 ab	73 a
25-29	13	67	82 b	77 a
30+	18	53	72 c	47 c

(a vs b, b vs c)  $\chi^2$  test;  $P < 0.01$ . (a vs c)  $P < 0.001$  in the same column

At present, there are several factors that are not fully-understood that control folliculogenesis, follicle growth, oocyte maturation, ovulation and fertilization. Further advances in understanding their functions and interrelations will allow greater efficiency in hormonal multiple ovulation treatments, and lead to reduced costs and greater benefits from this technique.

#### **4. Induced ovulation in recipients and estrus synchronization between donor and recipient**

Synchronization of estrus in recipients by means of progestagen treatment is performed at the same time as in donors. This is to ensure that both recipients and donors reach the same day of the estrous cycle at the time of embryo recovery and transfer.

Synthetic progesterone analogs, FGA (fluroggestone acetate) and MAP (medroxyprogesterone acetate), are usually used in pessaries (intravaginal sponges) for estrus synchronization. In sheep, 14 days treatment is required; in goats, progestagen treatment lasts 17 days. In Australia and New Zealand, use of progesterone administered vaginally using controlled internal drug release (CIDR) devices is very common.

Pregnant mare serum gonadotropin (PMSG) or equine chorionic gonadotropin (eCG) has FSH activity and to a lesser extent, LH activity. It is used at the end of the progestagen treatment to synchronize estrus. In ovine and caprine species, PMSG administration at sponge removal is recommended (Figure 1). PMSG dose varies according to breed and reproductive physiologic condition (indicative values: 200 to 400 IU).

#### **5. Donor fertilization**

Fertilization of female donors can be accomplished by natural service in the corral, or by artificial insemination (AI), using fresh or frozen semen. Siring in the corral is performed every 12 hours from the onset until the end of estrus.

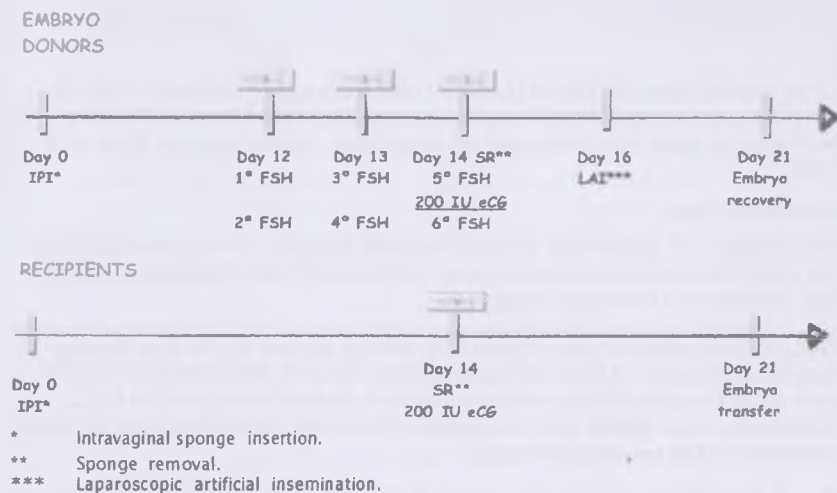


Figure 1. Hormonal treatment schedule for superovulation in Merino ewe donors and embryo recipients.

If AI is used, either with fresh or frozen semen, the laparoscopic procedure is recommended. In this way, semen is deposited in the uterine horns close to the fertilization site. This ensures an increase in the fertilization rate as well as a reduction in the insemination dose required.

### Insemination doses

The following are reference insemination doses for AI using fresh semen (in millions of spermatozoa):

- Cervical AI: 800 (sheep) - 400 to 600 (goats).
- Laparoscopic AI: 80 (sheep) - 100 (goats)

(Baril *et al.* 1989; Vallet and Baril 1990; Brebion *et al.* 1992).

In the case of frozen semen for laparoscopic AI, seminal dose in sheep (Wolff *et al.* 1994) and goats (Vallet and Baril 1990) is 100 million spermatozoa.

### Optimum time of AI

In sheep, laparoscopic timed AI with fresh semen is performed 32 hours after the onset of estrus (Brebion *et al.* 1992).

In the case of frozen semen, laparoscopic timed AI is carried out 40 to 55 hours after the end of progestagen treatment (Evans *et al.* 1986). In our experience with laparoscopic AI after estrus detection (Photo 2), ewes detected on heat 24 hours after pessary removal are inseminated 24 hours later; those showing heat 36 or 48 hours after sponge withdrawal, are inseminated 12 hours later.

In the caprine species, laparoscopic AI with fresh semen between 20 to 24 hours after the onset of estrus is recommended (Vallet and Baril 1990). If frozen semen is used, AI is performed 46 hours after sponge removal (Fieni *et al* 1990).

### **Fertilization rate**

The efficiency of fertilization in multi-ovulated females varies greatly depending on the fertilization technique used, the time of AI and the individual ovulatory response to hormonal treatment.

Oocyte fertilization can be achieved by natural service, cervical or laparoscopic AI. However it must be remembered that with natural service or cervical AI, in the case of high ovulatory response donors (more than 10 to 12 ovulations), a low fertility rate is obtained. This is due to the reduction of sperm transport in the reproductive tract.

Timed AI programs using laparoscopy and frozen semen for multiple ovulation treatments are very risky: they must only be used when the distribution of estrus for a specific population is known, considering the hormonal regime and the time of year. Fertilization efficiency of timed AI using frozen semen has presented variable results. Although Armstrong and Evans (1984) reported a 50% fertilization rate, in our experience the use of GnRH led to higher fertilization percentages. By administering a GnRH analog (8 µg buserelin, Receptal), at 36 hours after removal of sponges, we obtained fertilization rates of 70-80%, irrespective of whether AI was carried out at 42 or 55 hours after pessary withdrawal (Wolff *et al.* 1994).

However, laparoscopic insemination after heat detection is initially recommended. In our experience with the Merino breed, we carry out AI using frozen semen after estrus detection and with a total dose of 100 million spermatozoa per female. Ewes detected on heat at 24 hours after pessary removal are inseminated 24 hours later; while those in heat after 36 or 48 hours, are inseminated 12 hours later. In this way, we achieve fertilization rates close to 80%.

In goats, fertilization rates of 32% (cervical AI) and 65% (laparoscopic AI) using fresh semen were obtained (Moore and Eppleston 1979), while frozen semen using laparoscopy yielded 70-75% fertility (Fieni *et al.* 1990).

Fertilization rate is very dependent on ovulatory response to stimulation. In Alpine and Saanen goats, fertility diminishes when ovulatory response is high (>15 corpora lutea, 49% vs <15 corpora lutea, 66%) (Baril *et al.* 1989). The same results were observed in dairy sheep (Brebion, unpublished).

Artificial insemination fertility in multi-ovulated females is lower than that for non-treated control animals (Moore and Eppleston 1979; Armstrong and Evans 1984). Fertilization rate cannot be increased by more inseminations or by increasing sperm concentration in the seminal dose (Vallet *et al.* 1991; Brebion *et al.* 1992).

## 6. Embryo recovery

The methodology used to recover embryos consists of injecting a liquid medium that produces a flushing current through the uterine horns. A commercial medium based on phosphate buffer solution (PBS) with 10% inactivated adult bovine, sheep or goat serum is used. The serum can be obtained by aseptic blood sampling, using sterile material. Serum is centrifuged at 2000g for 15 minutes. The supernatant portion is then removed and centrifuged a second time. It is then filtered through a 22  $\mu$ m membrane. Inactivation of the complement protein is performed in a water bath at 56°C for 30 minutes. The resulting inactivated and filtered serum can be kept for one year at -20°C.

In sheep, embryo recovery is carried out on days 7 or 8 after pessary withdrawal. As initial embryo development in goats is delayed 12 to 24 hours, embryos are collected on days 8 or 9 following sponge removal (Table 3).

Embryos are collected on the days specified for the following reasons:

- Embryos are situated in the upper third of the uterine horns.
- They show a zona pellucida, necessary as a sanitary barrier.
- Freezing or vitrification is performed at the compacted morula or blastocyst stages.

Embryo recovery techniques in small ruminants maybe surgical or non-surgical.

Such procedures are carried out under general anesthesia. It is essential that the animals receive neither food nor water for 24 hours prior to surgery. An intramuscular tranquilizer (2 mg/10 kg xylazine 2%) and endovenous anesthetic (50 mg/10 kg sodium thiopental) are applied. It is also possible to use a combination of xylazine (2 mg/10 kg xylazine 2%) and ketamine (25 mg/10 kg ketamine hydrochloride) both administered intramuscularly (0.4 and 2 cc, respectively). Furthermore, a local anesthetic is applied to the surgical area (1 cc lidocaine 2%).

### *Surgical technique*

The female is placed head downwards on an inclined stretcher. The surgical area is shaved and disinfected. A midline laparotomy of 5-7 cm and 3 cm in front of the udder is carried out.

**Table 3.** Comparative embryo development in sheep and goats at different times after removal of intravaginal sponges (Adapted from Moore 1980).

Days after removal of sponges	Sheep	Goats
7	Morula	Morula
8	Compacted morula Blastocyst	Morula
9	Expanded blastocyst Hatched blastocyst	Compacted morula Blastocyst Expanded blastocyst
10	Hatched blastocyst	Hatched blastocyst

Before starting embryo recovery, ovulatory response is determined (corpora lutea count), either by exteriorization of the ovaries or by laparoscopic observation. Embryo recovery rate is assessed by dividing the number of collected embryos by the number of corpora lutea.

Embryo recovery consists of placing a catheter (K33) with a blunt needle at the end (50/20), with two lateral and one central orifice. A puncture in the utero-tubal junction is performed and the catheter is threaded inside the lumen of the uterine horn (1 cm), fixing it in place with a vascular clamp or ligature (Photos 3 and 4). A second puncture is carried out approximately 2 cm from the uterine horn bifurcation in order to inject 20 cc of PBS at 38°C (Photos 5 and 6). This produces a current that flushes towards the utero-tubal junction, where the catheter has been placed. The flushing medium is recovered in a previously warmed sterile Erlenmeyer (Photo 7). The same procedure is performed in the other uterine horn.

Once embryo recovery is finished, surgical incisions are sutured and antibiotics are administered.

A Foley catheter can also be placed in the uterine horn bifurcation and the flushing medium collected by the catheter itself.

The collected medium is poured into petri dishes and embryo identification is carried out under a magnifying glass (10x).

The first embryo recovery by surgical techniques can yield an average efficiency of 60 to 70%. The drawback to this procedure is the formation of post-surgical adhesions, which reduce the efficiency of subsequent embryo recoveries. We have obtained yields of 66, 41 and 35% for the first, second and third surgical procedures.

#### ***Non-surgical technique***

A non-surgical or laparoscopic technique was developed by McKelvey *et al.* (1986) in sheep and by Vallet *et al.* (1987) in goats. Three punctures (using trocars) are performed in the abdominal wall. The laparoscope is introduced through the first puncture. A three-way probe is introduced through the second puncture (the three ways correspond to: 1) input of PBS, 2) outlet of PBS and 3) insufflation of balloon). The third puncture is used for a non-traumatic clamp. This clamp permits reproductive tract manipulation, to advance the probe through the uterine lumen, and also to fix the utero-tubal junction during the PBS flow.

This technique requires a skilled operator and is expensive as a laparoscope is indispensable. Its yield of recovered embryos is lower (60%). The obstruction of the probe by clots or mucus can cause considerable difficulties. But the advantage is that it reduces adhesion formation and consequently, the percentage of recovered embryos does not decrease with subsequent operations. Vallet *et al.* (1987) recorded yields of 59, 58 and 68% in goats.

The average time required for embryo recovery is around 15 to 20 minutes (surgically) and 20 to 30 minutes (by laparoscopy) per animal.

Whichever technique is used for embryo recovery, if it is necessary to make sure that donor females do not become pregnant because of non-recovered embryos the administration of prostaglandin F<sub>2</sub> alpha (50 µg cloprostenol) after the interventions is recommended.

## **7. Identification of embryos**

The collected medium is poured into petri dishes and embryo identification is carried out under magnification and on a thermal plate at 38°C (Photo 8). A second inspection of the petri dish is always recommended. As they are identified, embryos are aspirated with a micropipette and placed in a small petri dish containing a preserving medium enriched with 20% serum, protected from light exposure and at laboratory temperature. Once the identification is finished, assessment of embryos is carried out. If possible, use a fume hood and filtered air. It is important to remember that these activities must be performed under strictly sterile conditions.

## **8. Assessment of embryo quality**

Embryo assessment is carried out based on morphological aspects and under a magnification of 10 to 40x. To observe the embryos from different angles, they can be moved using a micropipette or fine pipette. The integrity of the zona pellucida and its sphericity must be observed. Embryo development must correspond to that determined by its date of collection. A 24 hour delay is tolerable (Figure 2). Cells must be clear and present regular boundaries; opacity, if present, indicates degeneration.

In some embryos, partial detachment of cells in the perivitelline space can be detected. This characteristic is tolerable if the remainder constitute a uniform cellular mass, without opacity. When embryo merit is doubtful, a second evaluation is performed two hours later. This type of morphological examination does not provide an absolute viability test for embryos. However, significant embryo survival differences (Bari *et al.* 2003) (Table 4) were found when only regular quality embryos are transferred compared with good or excellent quality embryos (Annex 1). This difference is greater for embryo transfer of frozen or vitrified embryos.

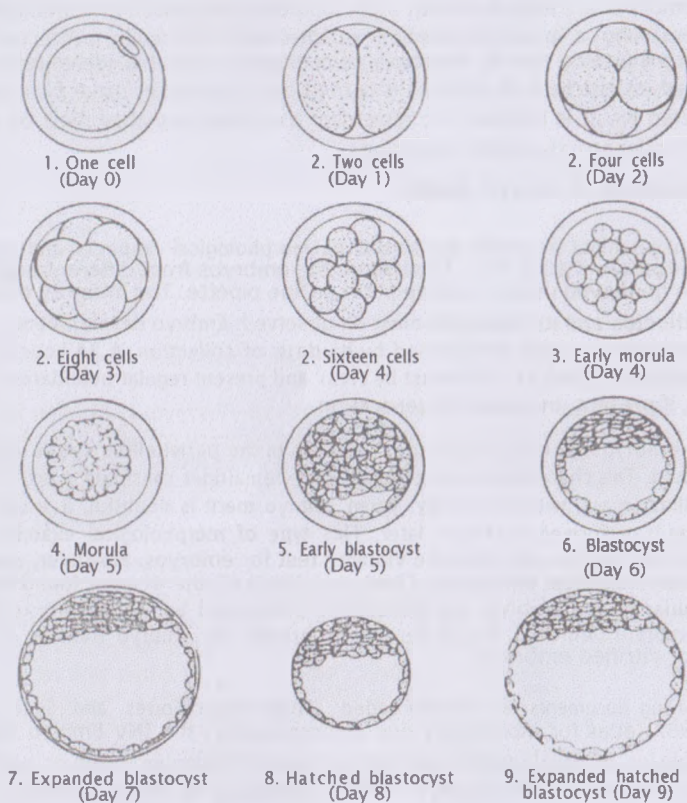
The following documents are recommended: Winterberger-Torrès and Sevellec's (1987) atlas for morphology and embryo quality, the IMV Embryo Atlas for examples of development and embryo quality in bovine embryos and the blastography of early development of the superovulated bovine embryo.

Embryo survival rate is not affected by day of embryo recovery (day 5 or 6 post estrus). Embryos that are at the normal stage of development for the collection day (day 5, morula; day 6, blastocyst), show similar survival rates (74%) (Bari *et al.* 2003). However blastocysts collected on day 5 show a high survival rate when compared with retarded morulae collected on day 6.

**Table 4. Survival rate for embryos of different quality grades in Scottish Black-face breed ewes (Bari *et al.* 2003).**

Embryo grade	Transferred embryos (n)	Embryo survival (%)
Grade 1	825	75.6 a
Grade 2	550	73.8 a
Grade 3	114	61.4 b
Grade 4	24	37.5 b

Different letters indicate statistical differences ( $\chi^2$  test;  $P < 0.05$ )



**Figure 2. Schematic chronology of embryo development in sheep (days post-estrus). The code for stage of development is numeric. Number 1 identifies an unfertilized oocyte or a one-cell embryo. Number 2 identifies embryos with two to 16 cells (approximately day 2 to day 5). Number 3 identifies an early morula, and numbers 4 through 9 identify post-compaction-stage embryos as illustrated above. In commercial embryo transfer, embryos usually are collected on days 6 to 8 of the estrus cycle (morula or blastocyst).**



## 9. Embryo transfer

Embryo transfer must take place immediately after collection and, in no case, must embryos be more than two hours in the preserving medium.

For frozen embryos, the time lapse between thawing and transfer is reduced to 20 to 30 minutes.

Embryo transfer is usually performed in the uterine horn ipsilateral to the corpus luteum.

Transfer of embryos is mostly accomplished by two alternate procedures: either surgical, or non-surgical by laparoscopy (González *et al.* 1991). In both cases, a puncture is performed on the dorsal side of the uterine horn and in its upper third portion. The embryos are located in the uterine lumen by means of a micropipette (conditioned in 10 µl PBS medium).

There is also a combined technique whereby the uterine horn is visualized using laparoscopy. A small 1 cm incision is made in the abdominal mid-line and the uterine horn is exposed using a clamp, so as to carry out embryo transfer (semi-surgical embryo transfer) (Photos 9 and 10).

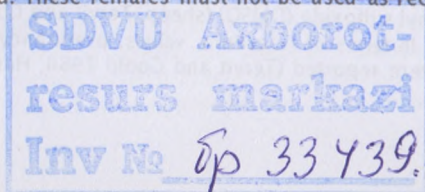
In dairy goats, a higher rate of embryo survival was found after transfer of two embryos per recipient (Moore and Eppleston 1979; Armstrong *et al.* 1983; Ter-vit *et al.* 1983). In sheep, global efficiency (lambs produced/transferred embryos) is greater when one embryo per female recipient is transferred (Cseh and Seregi 1993).

Tolerance in estrus synchronization times between donor and recipient females is  $\pm 1$  day. When donor-recipient estrus synchronization is optimal, embryo transfer efficiency is increased (Rowson and Moor 1966).

It is important to bear in mind the so called 'donor effect', defined as the variability observed in embryo survival rates (0 to 78%) for embryos of the same quality, from different mothers (Heyman *et al.* 1987).

Laparoscopic or visual ovary examination of recipients must be performed to ensure that females with one or two corpora lutea corresponding to days 6 or 7 of the estrus cycle are available. In addition, when recipients are selected, it must be remembered that embryo survival is influenced by the number of corpora lutea. Armstrong *et al.* (1983) reported embryo survival rates of 52, 63 and 75% for recipients with 1, 2 or 3 corpora lutea, respectively. Laparoscopic techniques contribute to a good classification of recipients based on their ovulatory response.

On certain occasions, especially in goats, recipients with cystic follicles or regressed corpora lutea are found. ~~These females must not be used as recipients.~~



Cervical embryo transfer is rarely used owing to difficulties in transposing the cervix (Lewalski *et al.* 1991; Flores-Foxworth *et al.* 1992).

It is very important to take into account the time interval between embryo recovery, identification and assessment of embryo quality and the corresponding embryo transfer. Because of the hard work involved in carrying out an ET program, it must be very well organized and coordinated to ensure optimal results.

## 10. Embryo preservation

### *Preservation by cooling to 5°C*

Embryo preservation permits the distribution of high-value genetic material on a local or international scale. For short distance transportation, refrigeration at 5°C ensures preservation for 24 hours in culture medium (ovum culture medium). Cooling rate is 1°C per minute.

Embryo warming is performed at 0.6°C per minute, or alternatively, by placing embryos directly in enriched PBS at 37°C. Embryos are examined under the magnifying glass, selected and transferred immediately. Survival rate varies between 35 and 48% (Bilton and Moore 1976; Driancourt *et al.* 1988).

### *Preservation by freezing in liquid nitrogen*

In domestic animals, the first results were published in the 1970s (Whittingham *et al.* 1972; Wilmut and Rowson 1973). In 1976, the first results from goats (Bilton and Moor 1976) and sheep (Willadsen *et al.* 1976) were published.

As with the semen of these species, freezing of their embryos and preservation in liquid nitrogen for long periods is possible. This method of preservation has made international trade feasible, and consequently, genetic material can be distributed at the global level.

The process involves submitting embryo cells to cryoprotectant media. These compounds (polyalcohols) penetrate cells by simple diffusion and are used to inhibit the formation of ice crystals by lowering the freezing point. The medium containing the embryos (extra-cellular) is richer in water than the intracellular medium. Consequently, at the start of freezing, the first ice crystals are formed in the extra-cellular medium. This, in turn, determines the diffusion of the intracellular water outwards and a corresponding reduction in intra-cellular ice formation. The time involved and freezing rate will determine subsequent embryo viability.

In both species - sheep and goats - greater embryo survival has been recorded during thawing when ethylene glycol was used rather than glycerol or dimethyl sulfoxide (DMSO) (sheep, Tervit and Goold 1984; goats, Le Gal *et al.* 1993). In terms of reference values, pregnancy percentages between 39 and 55% were reported (Tervit and Goold 1984; Hunton *et al.* 1985; Le Gal *et al.* 1993).

Freezing is performed with embryos of excellent or very good quality, and at the compact morula or expanded blastocyst stages (days 6 or 7 post estrus). The blastocyst stage is more resistant to freezing as even if some of its cells suffer severe damage future development is not compromised.

Embryo assessment before freezing is of vital importance; their state determines survival after thawing. Blastocysts without a zona pellucida can be frozen without affecting survival. In this case, its state of health constitutes the limiting factor.

Embryo preservation in liquid nitrogen can result from either freezing or vitrification techniques.

### Freezing Technique

Once recovered, embryos are classified and placed in successive 10 minute baths each with increasing ethylene glycol content (0.5, 1 and 1.5 M) (Annex 2) in PBS and 20% fetal bovine serum, at room temperature. During this period, cell shrinkage due to loss of water takes place, followed by slow re-expansion due to cryoprotectant entry.

When this stage has finished, they are placed in 0.25 ml straws. It is important to label straws carefully, indicating female donor, breed, number of embryos and date. Embryos are placed with 1.5 M ethylene glycol in PBS, separated at both ends with an air space and PBS + serum column. The straw is then sealed with polyvinyl alcohol.

Renard *et al.* (1982) showed the possibility of using two fractions containing 0.25 M sucrose in PBS at both ends of the straw. In this way, embryos in PBS + 1.5 M ethylene glycol are lodged in a central chamber (separated by air chambers from the remainder). Once thawed, the straw is shaken in order to join the fractions. Embryos are not observed under magnification, instead the whole content of the straw is transferred to the female recipient. This procedure is rapid and provides acceptable embryo survival (55 to 65%) (Tervit and Goold 1984; Hunton *et al.* 1985; Heyman *et al.* 1987; Le Gal *et al.* 1993).

Once embryos are conditioned, straws are placed in a programmable freezer; alternatively, they can be frozen manually. In the latter procedure, a steel cylinder is required in which to place straws together with a temperature sensor. The cylinder is fixed to a perforated rod (with a T crossbar), to enable gradual descent of the set into the thermos containing nitrogen.

The time elapsed between collection and start of freezing must not exceed 40 minutes.

The cooling rate is 1 to 3°C per minute, until -7°C. After 30 seconds at this temperature, seeding (induced crystallization) is carried out. Seeding is performed by touching each edge of the air fraction located above the column containing the embryos with a clamp cooled in liquid nitrogen for 2-3 seconds.

This induces early formation of ice-crystals which lowers the cooling rate, giving cells more time to dehydrate thus minimizing cell damage (de la Vega and Wilde 1991).

Subsequently, the temperature is kept at  $-7^{\circ}\text{C}$  for 10 minutes (time to reach equilibrium), and then a cooling rate of  $0.3^{\circ}\text{C}$  per minute is maintained until a temperature of  $-35^{\circ}\text{C}$  is reached. Stabilization time at  $-35^{\circ}\text{C}$  is 15 minutes. Finally, the straws are placed in the liquid nitrogen thermos and submerged in this medium at  $-196^{\circ}\text{C}$ .

### Thawing Technique

Thawing of the embryos is carried out in a thermostatic water bath at  $37^{\circ}\text{C}$  for 30 seconds. Then cryoprotectant extraction is carried out progressively in successive stages (5 to 10 minutes each), by submerging the embryos in petri dishes with decreasing concentrations of ethylene glycol (1 and 0.5 M) in PBS + 20% serum and, finally, placing them in a dish containing PBS + serum. The embryos are then evaluated based on morphologic characteristics. During this examination, embryos are selected according to damage due to the freezing/thawing process. As a reference value, from 10 to 30% of damaged embryos is acceptable.

Another technique for cryoprotectant removal after thawing consists in using sucrose. This substance, due to its high molecular weight, cannot penetrate the embryos. In this way, a hyperosmotic extracellular medium is generated which produces massive diffusion of the cryoprotectant outwards from the embryos. Furthermore, it produces water retention in the extracellular environment, preventing its ingress faster than the outflow of the cryoprotectant. In practice, thawed embryos are placed in a 0.25-0.5 M sucrose in PBS + serum solution for 5 to 10 minutes, followed by 3 successive 5-10 minute baths through PBS + serum.

In the case of purchased embryos, the corresponding freezing and thawing protocol as well as the health certificate should be requested from the supplier.

For sheep, percentages of viable embryos after thawing are higher for blastocysts compared to morulae (67 vs 31%) (de Paz *et al.* 1994). In goats, a higher survival rate for embryos at the expanded blastocyst stage or without a zona pellucida has been observed (Chemineau *et al.* 1986; Li *et al.* 1990). One drawback to the freezing of embryos without a zona pellucida is the lack of sanitary protection.

In sheep, we have used the slow freezing technique in a cylinder placed over nitrogen vapors. Embryos were packed in straws, in a central chamber containing 1.5 M ethylene glycol in PBS + serum, and having 0.25 M sucrose fractions at both ends of the straw. Thawing was performed rapidly in a water bath and fractions were joined in a petri dish to classify embryos under magnification. Then two embryos were transferred immediately to each female recipient. In this way, we had pregnancy rates of 30 to 40%.

In goats, despite using a freezing technique similar to that described for sheep, we found better results by placing embryos in PBS enriched with 0.4% bovine serum albumin (BSA) and by thawing in a 0.25 M sucrose solution in PBS, followed by 3 successive baths through PBS for 5 to 10 minutes. Two embryos per recipient were transferred, obtaining a pregnancy rate of 33%.

### Vitrification Technique

Vitrification is another technique for preserving embryos at lower temperatures. The physical principle is based on submitting embryos to a high cryoprotectant concentration in very low solution volumes, thus avoiding the formation of ice crystals.

The vitrification procedure is performed at room temperature (25°C), using 3 successive immersions of embryos in solutions containing increasing concentrations of glycerol and ethylene glycol in PBS with 20% fetal bovine serum (Traldi *et al.* 1999; Martinez *et al.* 2006). Briefly, embryos are submerged in: 1) glycerol 10% for 5 minutes, 2) glycerol 10% + ethylene glycol 20% for 5 minutes and 3) glycerol 25% + ethylene glycol 25% for 30 seconds (Annex 3). Then, the embryos are aspirated into tips with 1 µl medium (2 embryos/tip) and submerged in CryoTubes with liquid nitrogen (Gibbons *et al.*, 2008, 2009).

Devitrification is performed 'in air' at 37°C for 6 seconds. Embryos are immediately placed for 5 minutes in a solution of glycerol 12.5% + ethylene glycol 12.5% + 0.5 M sucrose in PBS with 20% bovine fetal serum. They are then placed at room temperature in two solutions of 0.5 and 0.25 M sucrose (5 minutes per solution). Finally, embryos are washed twice in PBS + serum (2.5 minutes per solution) (Gibbons *et al.* 2008) (Annex 3). Using this methodology in vitro, we obtained an embryo protrusion rate of 50% for morulae and 81.6% for blastocysts in sheep (Gibbons *et al.* 2008), and of 61% for blastocysts in goats (Gibbons *et al.* 2009). In vivo, in the ovine species, we registered an embryo survival rate of 42% (morulae) and 47% (blastocysts), and a pregnancy rate of 50% for both embryonic stages (2 embryos/recipient) (Gibbons *et al.* 2010). In goats, for blastocysts, we obtained embryo survival rates from 64 to 70% and pregnancy rates between 64 and 86% (2 embryos/recipient) (Traldi *et al.* 2009; Gibbons *et al.* 2010). This technique is not recommended for the vitrification of goat morulae owing to low reproductive efficiency.

## Reproductive Efficiency in Embryo Transfers

The following are average reference efficiency values found in the different multiple ovulation stages and ET (conventional FSH treatment):

Average number of corpora lutea per female donor	-	sheep 11, goats, 14-15
Embryos + oocytes recovery rate	-	60-70% for both species
Fertilization rate in laparoscopic AI	-	sheep 80%, goats 75%
Selection rate of embryos for freezing	-	80-90% in both species
Selection rate of embryos after thawing	-	70-90% in both species
Pregnancy rate (direct transfer)	-	70% in both species

Number of offspring born per female donor (treated at random):

Immediate ET	sheep: 4 lambs/donor	sheep
	goats: 5 kids/donor	goat
Frozen ET	sheep: 2 to 3.2 lambs/donor	sheep
	goats: 2 to 3.6 kids/donor	goat

Results obtained at INTA Bariloche for Merino breed sheep, and Angora and Criollo goats are as follows:

Average number of corpora lutea	-	sheep: 13 (a) - 17.5 (b)
	-	Angora goats: 8.6 (b)
	-	Criollo goats: 16 (a)
Surgically recovered embryos	-	60% in both species
Pregnancy rate by immediate semi-surgical ET technique	-	sheep 64%, goats 60%
Pregnancy rate by semi-surgical ET of vitrified embryos	-	sheep 50%, goats 64%

(a) Treatment with 80 mg pFSH (NIH Folltropin-V) + 200 IU PMSG

(b) Treatment with 200 mg pFSH (NIH Folltropin-V) + 200 IU PMSG

## Embryo Splitting

The possibility of splitting embryos permits an increase in efficiency of 94 to 131% (lambs born per each 100 split embryos) (Gatica *et al.* 1984; Chesne *et al.* 1987). However, this technique requires the use of a costly micromanipulator, which limits its use. Splitting embryos also provides the interesting possibility of obtaining twin animals for genetic research.

Efficiency in pregnancy rate when transferring split and frozen embryos is low (5.6%) (Shelton 1992).

## Sanitary Guarantee in Embryo Transfers

In spite of existing health norms, the risk of introducing diseases through the incorporation of live animals is very high. ET reduces this risk considerably, as embryos present a natural barrier to bacteria and viruses (Stringfellow *et al.* 1991). In the case of mothers infected with bluetongue virus (BTV), it has been shown that embryos carrying no health risk can be obtained. Consequently, genetic material can be recovered from infected livestock.

Passive immunity provided by recipient mothers gives the fetus invaluable health protection, and even more so when embryos are exported to countries with diseases that are exotic in relation to their country of origin. Quarantine and transport costs, as well as the difficulties animals face in adapting to a new environment (climatic, feeding and health conditions), also gain multiple extra commercial benefits when ET is used.

The International Embryo Transfer Society (IETS) is dedicated to the interchange and diffusion of scientific advances in ET and related techniques. Its import-export committee disseminates technical and scientific information for the formulation of health regulations in embryo commerce, and has issued an important reference publication concerning general ET norms (International Embryo Transfer Society, 1990).

## General Conclusions

Embryo transfer can increase the number of offspring from a genetically superior female, resulting in an average of four offspring per multiple ovulation treatment. Recent advances in the reproductive efficiency of ET have increased the possibility of its being used in genetic improvement programs by furthering the distribution of sheep genes with high productive value. Future research will be required to reduce costs and increase the number of offspring per donor sheep. This will facilitate its commercial application, as has already been accomplished with cattle.

There is no doubt at present that, for health reasons, ET is the safest method for importing various high production biotypes. The growth of international commerce in genetic material through the use of ET has proved the importance of the technique in providing health guarantees against exotic diseases and, furthermore, as a tool for the improvement of animal production.

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## ANNEXES

### Annex 1. Grades of Embryo Quality

Grade I, Excellent, ideal embryo, spherical, symmetrical, with cells of uniform size, color and texture. Embryo development corresponds to day of embryo recovery. No visible defects. Blastomeres are clearly visible and zona pellucida is intact.

Grade II, Good, some trivial imperfections, embryo has very few detached blastomeres from cellular mass and/or has a small quantity of vesicles. Its shape can be slightly irregular.

Grade III, Fair, embryo has defined defects: cellular detritus, irregular shape, very dark or very light color and/or slight cracking in zona pellucida. Few degenerate cells, vesicles and presence of detached blastomeres.

Grade IV, Poor, embryo shows severe defects: those corresponding to Grade III plus retarded development, serious rupture in zona pellucida - embryo may be partially displaced from it - very asymmetric form, tendency to disintegrate with granulation and vacuole formation in blastomeres. Comprises stages up to 8 cells and degeneration. This embryo grade is not transferable.

### Annex 2. Freezing Media

To prepare a 1 M solution of ethylene glycol, add 5.59 ml to 100 ml PBS with 20% fetal bovine serum. Simple calculation indicates ethylene glycol proportions for 0.5 and 1.5 M concentrations.

To prepare a 0.25 M sucrose solution, add 8.56 g sucrose to 100 ml PBS with 20% fetal bovine serum.

### Annex 3. Vitrification Techniques (Gibbons *et al.* 2008, 2009)

#### Vitrification solutions

##### *Solution 1*

4.5 ml Solution A\* + 0.5 ml glycerol (10%)  
5 min at 25°C

##### *Solution 2*

3.5 ml Solution A + 0.5 ml glycerol (10%) + 1 ml EG (20%)  
5 min at 25°C

##### *Solution 3*

2.5 ml Solution A + 1.25 ml glycerol (25%) + 1.25 ml EG (25%)  
30 seconds at 25°C

## Warming solutions

### *Solution 1*

3.75 ml Solution A + 0.625 ml glycerol (12.5%) + 0.625 ml EG (12.5%) + 0.86 g sucrose (0.5 M)\*\*  
5 min at 25°C

### *Solution 2*

5 ml Solution A + 0.86 g sucrose (0.50 M)  
5 min at 25°C

### *Solution 3*

5 ml Solution A + 0.43 g sucrose (0.25 M)  
5 min at 25°C

### *Solution 4*

5 ml Solution A  
2.5 min at 25°C

### *Solution 5*

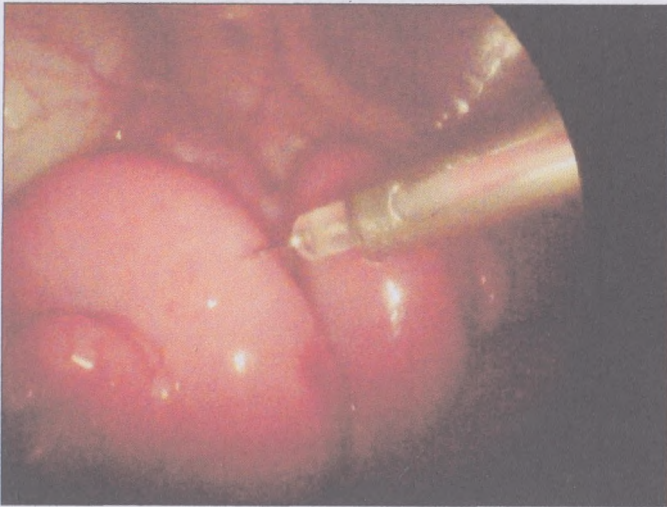
5 ml Solution A  
2.5 min at 25°C

\*Solution A: 28 ml PBS + 7 ml serum (PBS + 20% fetal bovine serum)

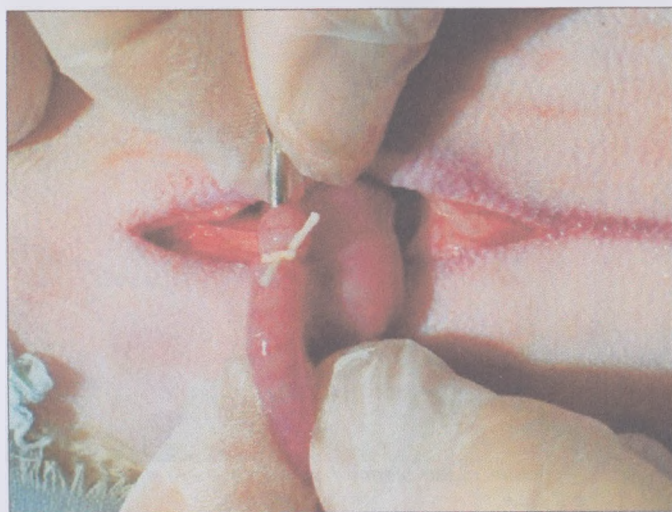
\*\*PM Sucrose: 342.296 g (For a solution 0.5 M = Add 171.148 g sucrose to 1000 ml solution)



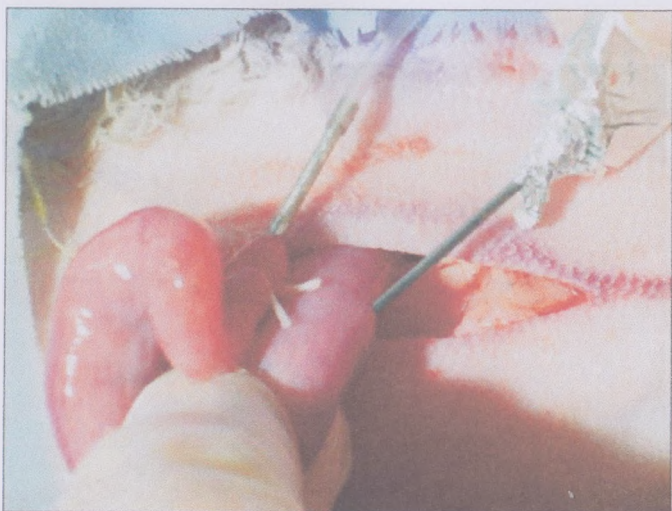
*Photo 1. Ovaries with multiple ovulation after treatment with 80 mg FSH and 200 IU PMSG.*



*Photo 2. Intra-uterine artificial insemination with frozen semen by laparoscopy in donor sheep.*



*Photos 3 and 4. Puncture and fixing of the catheter to the uterine horn (utero-tubal junction) for surgical embryo recovery.*



*Photos 5 and 6. Puncture of the uterine horn (distal third) and embryo recovery by current flow.*





*Photo 7. Recovery of embryos by current flow in the Erlenmeyer collector.*



*Photo 8. Identification and assessment of embryos under magnification.*



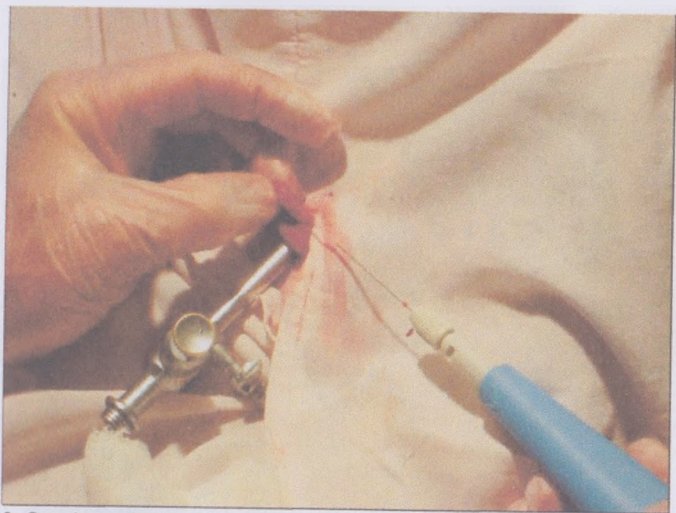
Photo 7. Recovery of embryos by current flow in the Erlenmeyer collector.



Photo 8. Identification and assessment of embryos under magnification.



*Photo 9. Puncture of the uterine horn (proximal third) for semi-surgical embryo transfer.*



*Photo 10. Semi-surgical embryo transfer with exteriorization of the uterine horn by clamping.*