



From cattle to sheep: a view of the difficulties and success of commercial *in vitro* production of sheep embryos

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Abstract

The *in vitro* production (IVP) of sheep embryos, unlike that for cattle, requires hormonal treatment with progesterone or a progestagen to improve efficiency of ovum pick-up (OPU). Another difference is that the process used to obtain oocytes is by OPU in cattle and LOPU (laparoscopic ovum pick-up) in sheep. Several factors affect oocyte recovery, including the skill of the technician, suction pressure and the aspiration system for LOPU. The LOPU technique is less invasive and can be performed weekly, biweekly or monthly in ewes. LOPU is an option to MOET (multiple ovulation and embryo transfer), mainly for animals of high genetic value that do not respond to the superovulatory treatment or only produce unfertilized oocytes. The embryos in the initial development stages can be transferred into the oviduct or into the cranial portion of lumen of the uterine horn, reducing the time for *in vitro* culture, which can be harmful to embryos. Although early results are promising, there are still many points to be improved in IVP of sheep embryos before it is used extensively in the commercial sheep industry.

Keywords: embryos, IVP, LOPU, oocytes, sheep.

Introduction

In the sheep industry today there is a growing concern about reproductive efficiency and genetic improvement of the animals, as well as the importance of efficient methods for assisted reproductive technologies, such as *in vitro* production of embryos using *in vitro* maturation (IVM) and fertilization (IVF). Animals of high genetic value that do not respond adequately to MOET (multiple ovulation and embryo transfer), or that have low rates of fecundity, may be optimized by using hormonal stimulation protocols and ovum pick-up, *in vitro* production of embryos (IVP), and transfer of embryos to recipients in the initial stages of development. Similarly, the use of frozen semen that results in low rates of fecundity following laparoscopic insemination could be optimized with the use of IVP.

The use of reproductive biotechniques, mainly IVP, improved the genetic value of cattle in Brazil during the last 10 yr. Due to the large demand, the technique was improved and better outcomes from IVP

resulted in improved pregnancy rates and better income for breeders. In sheep, commercial IVP has increased gradually during the last 6 yr. Different from bovine oocytes, which may be fertilized in dozens of laboratories in different parts of the country, there is only one laboratory for IVF/IVP for sheep in Brazil, and the length of time of transportation of the oocytes may directly affect the results.

The evolution of the technique may lead to better conception rates, as observed in Zebu cattle, making IVP commercially feasible for sheep.

From the field to the laboratory in the application of commercial IVP in sheep

Hormone treatments - Ovarian stimulation

In commercial IVP of small ruminants, different from cattle, animals are previously submitted to progesterone or progestagen priming using an intravaginal device, and this is accompanied by hormonal stimulation with FSH or a combination of FSH and eCG. These protocols enable synchronous recruitment of a larger population of follicles that can be aspirated by laparoscopic ovum pick-up (LOPU). Baldassarre *et al.* (1996) tested different protocols, from decreasing doses of FSH (control group) to a single administration of 80 mg NHI-FSH and 300 IU eCG administered intramuscularly 36 h prior to LOPU (One shot), but did not find differences in the recovery rate, cleavage rate or development of embryos to the blastocyst stage. Satisfactory results were obtained with this single injection of eCG protocol in cloning and transgenesis programs with goats (Baldassarre *et al.*, 2002, 2003) and for aged female goats of high genetic value (Baldassarre *et al.*, 2007).

The interval between ovarian stimulation and follicle aspiration is also important, and better results are accomplished when follicles are aspirated 36 to 48 h after the gonadotropin injection, without removing the vaginal device, in order to block ovulations (Baldassarre *et al.*, 2002, 2003; Morton *et al.*, 2005; Freitas and Melo, 2010). Cox and Alfaro (2007) recommended six applications of pFSH at 12 h intervals beginning 48 h after insertion of intravaginal progesterone releasing device and then LOPU 36 h after the last injection of pFSH.

In our commercial IVP programs, we have

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achieved good results with 200 IU FSH/LH (Pluset, Hertape Calier, Brazil) administered as four 50 IU doses of FSH along with a single dose of 125 µg d-cloprostenol (Ciosin, MSD, Brazil) with the first dose of FSH and 400 IU eCG (Folligon, MSD, Brazil) with the third dose of FSH (Fig. 1). Follicle aspiration is carried out

27-33 h after the eCG injection. This protocol was based on its effectiveness when used for cattle and sheep MOET donors, but with lower doses, since the objective is to recruit a larger number of follicles with diameters from 3 to 6 mm to *in vitro* maturation (IVM).

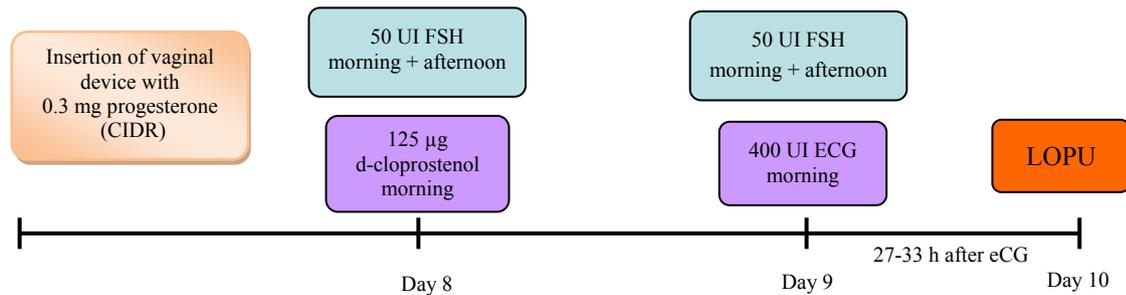


Figure 1. Protocol for hormone stimulation and follicle aspiration by laparoscopy (LOPU) in sheep. Source: Embryo Sys, Ouro Fino, 2012 (unpublished data).

The efficiency of the method depends on the protocol, the individual response to hormone stimulation, and the periodicity of LOPU (Baldassarre e Karatzas, 2004; Cognié *et al.*, 2004; Morton *et al.*, 2005).

LOPU - The differences between sheep and cattle

Although manipulation of the ovaries was considered a limiting factor for follicular aspiration in small ruminants compared to bovine species (Baldassarre *et al.*, 1994, 1996), and despite the fact that the first ovine IVF results were from ovaries from a slaughterhouse (Crozet *et al.*, 1987; Cognié *et al.*, 2004) the development of an aspiration system adequate to small ruminants and LOPU has enabled a leap in knowledge and made IVF feasible.

Suction pressure, the aspiration circuit, the diameter and length of the puncture needle, the type of pump and the ability of the laparoscopist, directly influence success of oocyte recovery (Rodriguez *et al.*, 2006; Gibbons *et al.*, 2008). Pressure ranges from 25 to 100 mm Hg (Baldassarre *et al.*, 1996; Cognié *et al.*, 2004; Cox and Alfaro, 2007; Morton *et al.*, 2008), 10 to 20 ml H₂O/min (Cognié *et al.*, 2004) or 50 to 70 drops/min (Baldassarre *et al.*, 2003). From a practical standpoint, quick and continuous dripping in the oocyte collecting tube is the best parameter for the evaluation of vacuum pressure. Puncture needles range from 18 to 21 gauge, with recovery rates of 40 to 90% (Cognié *et al.*, 2004; Bernardi, 2005; Morton *et al.*, 2005; Rodriguez *et al.*, 2006). From the pool of oocytes aspirated, 60 to 80% were selected for *in vitro* maturation (Cognié *et al.*, 2004; Bernardi, 2005). In 587 sessions of commercial ovine LOPU, Basso *et al.* (2008) achieved an average of 14.3 oocytes per aspiration.

Development of equipment with Brazilian

technology (WTA- Watanabe Tecnologia Aplicada Ltda, Cravinhos, SP, Brazil) made LOPU easier. A transparent plastic device with a 22 gauge needle is attached to a stainless steel rod. The aspiration system goes through the rod in a way that makes it easy to see the flow and appearance of the follicular fluid during the oocyte recovery process.

In small ruminants, good sedation and adequate immobilization are essential for efficient aspiration. Commonly, the animal is placed in an inverted position on a cradle at a 45° angle, in order to prevent accidents when the trocar is inserted into the abdomen. Aspiration is more efficient if atraumatic grasping forceps are used to stabilize the mesovarium, making it possible for the technician to turn the ovary in different directions for better positioning, visualization, and follicle aspiration. At this point, the difference between the technique in sheep and cattle is considerable: manipulation of the ovary and the narrow rectal route in bovine species make the process more stable, and visualization and puncture of the follicles is guided by ultrasound.

As LOPU is a less invasive procedure when compared with laparotomy, the rate of formation of adhesions after continuous aspirations is not very high. According to Stangl *et al.* (1999), regardless of the hormone stimulation protocol used, ewes subjected to LOPU once a week for 10 weeks did not experience any negative effects on reproductive performance. Only small adhesions were observed between the omentum and the abdominal wall. Besides, if bleeding occurs, saline solution with heparin may be instilled to prevent the formation of adhesions (Baldassarre *et al.*, 2003; Cox and Alfaro, 2007).

Observation of the vessels on the follicle wall during laparoscopy make it possible to choose a less vascular site to introduce the needle, minimizing the loss of follicular fluid and oocytes, and aspirating



follicular fluid containing little or no blood, which is different from what occurs in cows subjected to OPU. The aspiration of follicles greater than 6 mm should be avoided, because they may be atretic or dominant. Follicles smaller than 2 mm do not achieve developmental competence *in vitro*; therefore, the technician should focus on selecting and aspirating those follicles that are able to achieve nuclear and cytoplasmic competence during *in vitro* maturation (Crozet *et al.*, 1987; Cognié *et al.*, 2004; Traldi, 2009; Gilchrist, 2011).

Despite of the easy visualization and counting of the number of aspirated follicles, follicular fluid and oocytes may be lost during the process of follicle puncture and during aspiration (Baldassarre *et al.*, 2002). The use of heparin is essential both in the aspiration medium and in the periodic washing of the circuit for flow of aspiration fluid to prevent coagulation and blockage of the system. In our study, we used 5 IU of sodium heparin/ml of buffered saline medium (DPBS). This solution is also employed to wash the

puncture needle under high pressure, in order to prevent loss of follicle contents if the circuit is blocked.

Classical studies of IVP in sheep demonstrated greater *in vitro* competence and nuclear and cytoplasmic maturation in oocytes from follicles that were 3 to 6 mm in diameter with uniform distribution of cumulus layers (Crozet *et al.*, 1987). However, oocytes with three or four layers of granulosa cells, which are the majority in the pool obtained from ovaries collected in slaughterhouses and in animals submitted to hormonal stimulation, are similarly competent for IVM (Baldassarre *et al.*, 2003; Capezzuto *et al.*, 2004; Bernardi, 2005). Oocytes should present a compact cumulus and evenly granulated cytoplasm (Baldassarre *et al.*, 2003; Cognié *et al.*, 2004; Bernardi, 2005). According to this oocyte selection, from 91 aspirations performed in Santa Inês, Dorper, White Dorper, and Lacaune sheep (Table 1), we obtained an average of 9.2 oocytes, from which 83% were selected for IVM, producing an average of 5 embryos on the 3rd day of culture.

Table 1. Results of follicle aspiration by laparoscopy (LOPU) in Santa Inês, Dorper, White Dorper, and Lacaune sheep submitted to a hormonal stimulation protocol.

Parameters	Breed			
	Santa Inês	Dorper	White Dorper	Lacaune
Number of aspiration sessions	45	23	11	12
Mean number of oocytes collected by aspiration	8.8	11.3	7.6	8.2
Mean number of oocytes used in IVM	7.3	9.2	6.4	7.1
Total number of embryos produced	180	174	55	54
Mean number of embryos produced by LOPU	4.0	7.6	5.0	4.5
Pregnancy rate (%)	36.7	18.4	23.6	25.9

¹Four doses of 50 IU FSH/LH in 12-h intervals + 125 µg of d-cloprostenol in the first FSH administration + 400 IU eCG in the third dose of FSH; aspiration carried out 27-36 h after the eCG dose. Source: Embryo Sys, Ouro Fino, 2012 (unpublished data).

Transportation of oocytes: from the field to the laboratory

The length of time for transport of oocytes from the field to the laboratory may range from some hours to longer intervals, such as when long distance air or land transportation is necessary (Max *et al.*, 2012). In order to prevent changes during maturation of oocytes and to maintain the desired pH of the medium, oocytes are stored in maturation medium and placed in an oocyte transport device (WTA-Watanabe Tecnologia Aplicada Ltda, Cravinhos, SP, Brazil) at constant temperature of 38.3°C, and under an atmosphere of 90% N₂, 5% CO₂, and 5% O₂. The time to arrival of oocytes in the laboratory should be no longer than 24 h after the first aspiration, considering that the length of IVM of sheep is 24 h, similar to that for bovine species (Crozet *et al.*, 1987;

Cognié *et al.*, 2004).

The recovery of oocytes by ovarian slicing may be used for high genetic value females culled because of old age, death due to accidents or during delivery of an offspring, or due to natural death (Traldi, 2009). Ovaries are placed in saline solution at 38°C and sent to the laboratory; after the aspiration of superficial follicles the ovaries are sliced with a scalpel blade and competent oocytes from follicles embedded within the ovarian stroma are retrieved.

Commercial IVP with a new point of view: from cattle to sheep

The IVP is an assisted reproduction technique widely used in cattle in Brazil. In the last few years it has started to be applied to sheep and goats, using the knowledge and methodologies established for IVP in



cattle. The use of this technology has some advantages, such as the multiplication of superior genotypes, mainly in ewes that do not respond adequately to MOET or that show low rates of fecundity.

Table 2 summarizes current information on IVP

embryos in cattle and sheep in Brazil. Bovine embryos are transferred at the blastocyst stage, while sheep embryos are transferred at the initial stages of embryonic development, from the 2nd to the 4th day after *in vitro* fertilization.

Table 2. Comparative data on collection of oocytes and production of embryos using *in vitro* fertilization in cattle and sheep.

Species	LOPU	Total of oocytes	Mean	Viable oocytes	Mean	Embryos	Mean	Conversion rate ¹
Cattle (2010-2012)	9,156	261,330	28.5	144,460	15.8	74,362	8.1	28.5%
Sheep (2006-2009)	553	6118	11.1	-	-	2,825	5.1	46.2%

¹Conversion rate: number of embryos/total of oocytes. Source: In Vitro Brasil, Mogi Mirim, 2012 (unpublished data).

According to a commercial embryo producing company in Brazil (In Vitro Brasil), oocyte/embryo conversion rates are lower in cows because they are cultured *in vitro* until the blastocyst stage, whereas sheep embryos are transferred in the initial stages of development into the oviduct on day 2 (2 to 6 cells), or into the lumen of the cranial portion of the uterus on day 4 (8 to 32 cells). The shorter time that embryos are in culture

medium minimizes possible changes both in the embryonic disc and in the trophectoderm, reducing the incidence of developmental anomalies (Traldi *et al.*, 2009; Table 3). At least five recipients are synchronized per donor from which oocytes are aspirated. One or more embryos may be transferred according to the number of corpora lutea, embryos available, stage of embryonic development and site of transfer into either the oviduct or uterus.

Table 3. Results of *in vitro* production of sheep embryos from March 2011 to March 2012, transferred in the oviduct (day 2) or uterus (day 4).¹

Donors	Total of oocytes	Oocytes in IVM	Embryos produced	Embryo/LOPU	Site of embryo transfer	Recipients	Pregnancy rate	Embryo survival/donor
133	1,803	1,767	916	6.9	Tube	500	61%	2.3
52	525	507	302	5.8	Uterus	237	30%	1.4

¹Day 2 and day 4: 2 or 4 days of *in vitro* culture. Source: Fábrica do Embrião, Campinas, 2012 (unpublished data).

In Brazil, the breed with the greatest demand for IVP is Santa Inês, followed by Dorper, White Dorper and Lacaune breeds, including lambs. The greatest demand in elite flocks of sheep is for older ewes that do

not produce adequate numbers of embryos in MOET programs. However, fetal survival rates in general are still low and current birth rate is lower than two lambs per aspirated ewe (Table 4).

Table 4. *In vitro* production of embryos from oocytes of adult ewes and ewe lambs.

Breed	No. LOPU	No. oocytes	Mean	Embryos	Mean	Conversion rate	No. recipients	Embryos/recipients	Pregnant recipients	Pregnancy rate	Birth per LOPU
Dorper	183	2,396	13	1,266	6.9	53.0%	534	2.4	201	38.0%	1.4
White Dorper	20	259	13	145	7.2	56.0%	58	2.5	23	40.0%	1.5
Santa Inês	350	3,463	10	1,414	4.0	41.0%	660	2.1	297	45.0%	1.1
TOTAL	553	6,118	11.1	2,825	5.1	46.2%	1,252	2.3	521	41.6%	1.3
Lambs	21	1,091	52	602	-	59.0%	178	3.4	25	14.0%	1.5

Source: In Vitro Brasil, Mogi Mirim, 2010; presented at the Practitioner's Forum: Cattle IVF in South America: a short history of great results; IETS 2010.

Traldi *et al.* (1999) demonstrated that vitrification is the most efficient technique for the cryopreservation for blastocysts of small ruminants produced *in vitro*, although the best rates of embryonic, fetal and postnatal survival have been obtained in goats rather than in sheep. We observed a

great difference in embryonic survival of fresh embryos (8 to 32 cells) compared with vitrified blastocysts from ewes (Table 5). In order to be commercially viable, conception rates, following use of both fresh and vitrified IVP embryos, must be improved.

Table 5. Conception rates following transfer of fresh and vitrified sheep embryos derived by *in vitro* fertilization.

Type of embryo	Transferred embryos	Pregnant recipients	Conception rate ¹
Fresh	463	125	27.0%
Vitrified	36	5	13.9%

¹Pregnancy diagnosis by ultrasound 50 days after transfer. Source: Embryo Sys, Ouro Fino, 2012 (unpublished data).

Final considerations

The first challenge of the technician is to convince the breeder to use this tool to add value to the herds or flocks, given the high cost of the service. This includes the technician's fee to aspirate oocytes from the donors and transfer embryos to recipients, and the laboratory fee to produce the embryos. Current fees range from US\$250 and US\$300 per pregnancy, divided equally between the technician and the laboratory. When we emphasize to the breeder the low rate of side effects and minimal risks of the LOPU in comparison to MOET, and the possibility of aspirating the most valuable females monthly, obtaining an average of 12 lambs/ewe/yr, the interest in the use of the biotechnique increases.

The option for site of transfer of embryos, i.e., oviduct or uterus, soon after the first cleavages shortens their time in culture and enables their development to continue in adequate, intrauterine conditions, and prevents long-term exposure to factors in the culture medium that may cause undesirable epigenetic changes. From such changes it may result abnormal fetal development associated with large offspring syndrome that is associated with the lack of signaling for parturition by the fetus or death due to respiratory insufficiency. Such undesirable outcome may take the breeder from excitement to a complete lack of interest and disappointment regarding the use of the technique (Traldi, 2009).

Despite of the considerable progress in commercial IVP in sheep, some challenges remain: the development of less costly protocols for stimulation of the ovaries, and greater rates of embryonic survival and postnatal viability, which are still low for offspring from IVP embryos of sheep as compared with cattle.

Conclusions

A number of issues need to be addressed in the ovine embryo IVP process in order to turn it into an economically viable technique that may be used as a tool for genetic improvement of the species. To accomplish this aim, ovine embryo IVP needs to allow the largest possible number of oocytes matured *in vitro* to cleave, develop beyond the stage of embryonic arrest and resume development until the time of blastocyst hatching *in vitro*, and mimic developmental events that happen within the intrauterine environment (Traldi *et*

al., 2009).

The growing number of technicians involved with this biotechnology may increase the exchange of information and expertise and increase the possibility that more laboratories become interested in IVF and IVP for small ruminants.

Sexed semen, reverse semen (thawed and sexed), and vitrification of embryos are tools that are used in IVP for cattle, but they are not used or they are used with limitations in sheep. However, these techniques open new frontiers for research, create challenges, and may ultimately lead to successful commercial IVP in the sheep industry.

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