



Advances and limitations of *in vitro* embryo production in sheep and goats

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Abstract

This review summarizes the latest advances and main limitations for the implementation of *in vitro* embryo production programs in sheep and goats. We describe the laparoscopic assisted technique for oocyte retrieval and propose new insights for follicular manipulation to improve oocyte quality. Further description of the routine conducted in our laboratory for the *in vitro* process of oocyte maturation, fertilization and embryo culture is presented, with emphasis in the main issues for the success of the technique. Protocols for fixed time embryo transfer (FTET) are proposed and the optimal number of *in vitro* produced (IVP) embryos to be transferred *per* female is discussed. In addition, we present pregnancy outcomes and birth rates recently obtained with FTET with IVP embryos cryopreserved by vitrification with new minimum volume methods. In summary, due to important refinements for *in vitro* embryo production in sheep and goats achieved in the recent years, this technology is now available for its implementation in commercial programs for genetic improvement, for the production of genetically engineered sheep and goats, and for basic research in reproduction.

Keywords: cryopreservation; ewe, goat, LOPU, superovulation.

Follicular aspiration by laparoscopy

The success of an *in vitro* embryo production (IVEP) program for either research purposes or industry application depends largely on the availability of a continuous number of good quality oocytes. Although slaughterhouse ovaries represent a very useful cheap and rich oocyte source for research projects, the application of this technology in the industry and livestock requires oocyte retrieval from live animals. Follicular aspiration by laparoscopy (laparoscopic ovum pick-up; LOPU) is the best available technique to obtain oocytes from sheep and goats, firstly described by Robin Tervit in New Zealand and Hernan Baldassarre in Argentina in the early 1990s (Tervit *et al.*, 1992; Baldassarre *et al.*, 1994). This approach has the advantage of eliminating the poor results observed with the traditional laparotomy technique, besides the possibility of repeating the procedure several times in the same female, and allowing the production of offspring from prepubertal animals (Baldassarre and Karatzas, 2004). LOPU is used to recover oocytes for conventional IVP, but also for the production of zygotes for the generation of transgenic founders (Baldassarre *et*

al., 2003b) or for propagating transgenic animals by somatic cell nuclear transfer (Baldassarre *et al.*, 2003a). Recently, this technique has also been proposed as a useful tool to assist the generation of genome edited sheep and goats (e.g. CRISPR system; Menchaca *et al.*, 2016).

During the LOPU procedure, the animal is restrained on a standard laparoscopy table under general anesthesia. In our laboratory, general anesthesia is induced with ketamine and diazepam by i.v. route and is maintained with isoflurane inhalatory anesthesia. LOPU technique is further described by H. Baldassarre (Menchaca *et al.*, 2016), and briefly, consist of a laparoscopy equipment with a 5 mm diameter and 0° angle telescope, two 5.5 mm trocar/cannula sets (one for the laparoscope and one for the forceps), one 3.5-5 mm trocar/cannula set for the aspiration pipette, a 5 mm atraumatic grasping forceps, a fiber optic cable and a light source. The oocyte aspiration set consists of a collection tube with an inlet connected through tubing to the aspiration pipette and an outlet connected through tubing to a vacuum pump. The aspiration pipette consists of a 20G short bevel needle glued to the tip of a 30 cm-long acrylic tubing, with a 3 mm external diameter and 1 mm internal diameter. The ovarian surface is exposed by pulling from the fimbria in different directions using the forceps, and the follicles are punctured one by one using the aspiration pipette. For a trained team, in average it takes about 15-20 min per donor (including preparation) and recovery rate of cumulus-oocyte complex (COC) ranges from 50 to 90%.

The possibility to use prepubertal females of 1-3 month old as oocyte source is another advantage of the LOPU technique, reducing the generational interval and accelerating the process of genetic improvement (Paramio, 2010). In addition, LOPU can be performed repeatedly in the same female with an interval between aspiration sessions of 4 days without affecting oocyte retrieval and quality (Gibbons *et al.*, 2007). These two possibilities remarkably increases the capacity of multiplication of a female. The incidence of injuries caused to the ovary after successive LOPU sessions has been under study. In general, most of the reports did not found severe ovarian lesions that interfere with follicular aspiration, oocyte retrieval or embryo yield after 3 to 7 repeated hormonal treatments and LOPU sessions (Pierson *et al.*, 2004; Gibbons *et al.*, 2007; Teixeira *et al.*, 2011; Sanchez *et al.*, 2014). Other authors reported the presence of small adhesions in around 30% of sheep after repeated LOPU over a 10-week period (McEvoy *et al.*, 2006). In our experience, although some adhesion or fibrosis may be eventually

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Received: May 15, 2016

Accepted: June 22, 2016



found in few animals after several follicular aspiration sessions, it does not represent a relevant limitation.

Manipulation of follicular growth

In order to recover a high number of good quality COCs per LOPU session, the ovarian status must be synchronized and stimulated using gonadotropins. For synchronization, different protocols have been used in both sheep and goats and most of them are based in estrus synchronization treatments, mainly with progesterone or progestogen-containing intravaginal devices (e.g. CIDR; sponges). Usually, the devices are applied during 9-11 days together with a luteolytic dose of prostaglandin (PG) F2alpha or an analog at the time of gonadotropin treatment start. After synchronization, several treatments have been proposed for follicular stimulation prior to LOPU, being the most popular the multiple FSH injection regime and the so-called oneshot regime, consisting of one dose of FSH plus equine chorionic gonadotropin (eCG) described by Baldassarre and colleagues (Baldassarre *et al.*, 2002).

Different studies in sheep and goats have shown that *in vitro* matured (IVM) oocytes obtained by follicular aspiration have lower oocyte competence when compared to those matured *in vivo* (Cognie *et al.*, 2003), suggesting unsuitable IVM conditions, but also low quality of the oocyte population obtained by aspiration. Oocyte competence is affected by the follicular size from which the COC is collected (Crozet *et al.*, 1995) and also by follicular wave dynamics, growth status and dominance further studied in cattle (Adams *et al.*, 2008). The ideal method should ensure a homogeneous pool of medium (~4 mm) and young follicles in growing or static phase containing healthy oocytes, and never in late atresia. With this in mind, recently we have tested new approaches for follicular control before LOPU in sheep. Previously, we had described the Day 0 protocol for stimulation of the first follicular wave, for superovulation in conventional embryo production or MOET programs (Menchaca *et al.*, 2002, 2007, 2009, 2010). This protocol synchronizes the ovulation (defined as Day 0 of the cycle) to induce the emergence of the first follicular wave 72 to 84 h after progesterone device removal and eCG administration. In Day 0 Protocol for MOET programs, FSH is administered in several doses (e.g. 6 or 8 doses) twice a day with the first dose given 84 h after device removal (i.e. soon after ovulation), coinciding with follicular recruitment of wave 1. Recently, we have validated this protocol associated to LOPU. In this case, a single dose of 80-100 mg of pFSH reconstituted in sodium hyaluronic acid (MAP-5, Vetoquinol) for slow releasing was given on Day 0 (i.e. 84 h after device removal). LOPU was performed 72 h after FSH dose (i.e. on Day 3). Preliminary data show higher number of aspirated follicles and collected oocytes using the Day 0 protocol with pFSH in MAP-5 when compared with those obtained from no FSH treated ewes (Cuadro *et al.*; IRAUy, Montevideo, Uruguay; unpublished results). Interestingly, the effect of pFSH in MAP-5 used in Day 0 protocol was

potentiated when an intravaginal progesterone releasing device (DICO 0.3 g) was inserted from Day 0 (i.e. at FSH injection) until the day of LOPU. Follicular development in the presence of high progesterone levels significantly enhanced COCs quality, oocyte fertilization rate, and embryo production (Cuadro *et al.*; IRAUy, Montevideo, Uruguay; unpublished results). The addition of an intravaginal progesterone releasing device during the follicular development seems to be recommended during the stimulation of wave 1.

In vitro maturation

The procedure for *in vitro* embryo production in sheep and goats has been extensively studied and is further described in previous reviews (Cognie *et al.*, 2004; Paramio, 2010; Souza-Fabjan *et al.*, 2014; Paramio and Izquierdo, 2014, 2016). For that reason, this document describes only the main features and weaknesses of this process.

Regarding IVM, probably most of the effort has been focused on improving the conditions of *in vitro* culture media. In that sense, a variety of media system have been studied, proposed and adopted. In general, most laboratories use TCM199 supplemented with different components for this stage. This medium can be supplemented with gonadotropins as LH, FSH or a combination of both since it increases the number of oocytes reaching MII and improves the rate of viable embryos (Moor and Trounson, 1977). In addition, cysteamine supplementation acts as a glutathione precursor, improving oocyte maturation clearly demonstrated in several species included sheep and goats (de Matos *et al.*, 2002; Cognie *et al.*, 2003). Estradiol is another proposed component to be included into maturation medium since it is present in the preovulatory follicular fluid. However, its effect is controversial (Guler *et al.*, 2000) and seems to be depending on the presence/absence of other components. Although in lower concentration than follicular fluid, estradiol is present in another component normally added to the IVM medium, the estrus sheep/goat serum. Estrus sheep/goat serum is routinely used in our laboratory, although its variability is well known, containing each batch different levels of metabolites, growth factors, hormones, proteins, lipids, etc., making it difficult to standardize culture conditions. In addition, as a biological component, serum represents a sanitary risk that may be relevant for commercial application of IVEP. On the other hand, chemically defined IVM media have greater repeatability and are safer from a sanitary point of view, deserving future investigation, validation and adoption. A deeper knowledge about the role of follicular environment in oocyte maturation, the function of its components and the interaction between the oocyte and cumulus cells, will help to improve IVEP in the future.

Probably, there are as many variations in embryo culture media as IVEP labs in the world. In our laboratory, maturation medium is composed of TCM-199 supplemented with estrus sheep serum, FSH, LH, cysteamine and antibiotics. Excellent and good quality



oocytes are incubated in groups of 25-30 per 100 μ l drops of IVM medium under mineral oil for 24 h at 39°C in a humidified atmosphere containing 5% CO₂. Normally, more than 90% of metaphase II oocytes is expected to obtain under these conditions.

***In vitro* fertilization (IVF)**

There are different strategies to prepare ovine/caprine semen for IVF, similar to those used in cattle. Motile spermatozoa are obtained by Percoll gradient or swim-up method and added to COCs in fertilization medium after IVM. Sperm capacitation is normally achieved with the use of heparin added to the fertilization medium and during semen preparation before IVF (e.g. during swim-up). In addition, ionomycin has been tested to improve fertilization rates in goats with good results (Urdaneta *et al.*, 2004).

Some laboratories remove cumulus cells at the end of IVM just before IVF (Cognie *et al.*, 2004). The effect of cumulus cells during IVF has been studied in sheep (Menchaca *et al.*, 2012) and goats (Souza *et al.*, 2013). We found a positive effect of cumulus cells during IVF, with greater cleavage rate (85 vs. 77%; $P < 0.05$) and developmental rates (37 vs. 17%; $P < 0.05$) when cumulus cells were maintained during IVF than when removed prior to IVF, respectively ($P < 0.05$). Similar improvement has been reported in goats with greater blastocysts yield (48 vs. 37%; $P < 0.05$) when cumulus cells were maintained during IVF (Souza *et al.*, 2013). After testing several protocols and components, in our laboratory we use synthetic oviduct fluid (SOF) supplemented with heparin, hypotaurine and estrus sheep serum, both for IVF medium and sperm preparation medium. Spermatozoa are capacitated by swim-up with an incubation time of 15 min, and an insemination dose of 1×10^6 total sperm per drop of 100 μ l SOF under mineral oil containing 20-30 oocytes. Oocytes and sperm are co-incubated for 18 to 22 h at 39°C in a humidified atmosphere containing 5% CO₂. Working under these conditions with frozen semen, processing more than 6,000 COCs during the last year, the cleavage rate at 48 h from insemination ranged from 80 to 90% in more than 80% of the IVP sessions.

***In vitro* culture (IVC)**

Four events are crucial during IVEP: cleavage, embryonic genome activation (8-16 cells), morula compaction, and blastocyst formation accompanied by the formation of the inner cell mass and trophoblast (Lonergan *et al.*, 2003). These events are negatively affected by inadequate culture conditions and several strategies have been designed to mimic the female tract in the lab, with approaches that include *in vivo* culture of zygotes on the oviduct of temporary recipients, *in vitro* co-culture with somatic cell support, and semi-defined media to suit embryo requirements (Paramio and Izquierdo, 2016).

Different culture media have been tested in small ruminant's embryos; however, SOF medium first described in the 1970s (Tervit *et al.*, 1972) is the most

used among laboratories to culture sheep embryos and has been adapted to numerous species including cattle, pigs and goats. In our experience, changing or refreshing IVC medium on Day 3 after insemination improved blastocyst yield on Day 6 from 33.6% (78/232) to 42.0% (102/243; $P < 0.05$), probably by providing fresh nutrients and removing toxic metabolites (Vilariño *et al.*, 2012). Usually embryos are maintained in groups during IVC since this improves blastocysts yields than those single cultured (Gardner and Lane, 1993). In our laboratory, IVC is performed in groups of 20-30 embryos in 100 μ l drops using SOF under mineral oil and supplemented with BSA, essential and nonessential amino acids and antibiotics, at 39°C in 5% O₂, 5% CO₂ and 90% N₂. Expected blastocyst rate under these conditions is around 30 to 40% (number of blastocysts on Day 6 from COCs in IVF).

Transfer of IVP embryos

Embryo transfer outcomes could be affected by the embryo quality and by intrinsic and extrinsic factors from the recipient (breed, age, reproductive status, nutrition and health). Regarding embryo quality, survival rate progressively increases with the stage of embryo development for *in vivo* derived embryos (Bari *et al.*, 2003). Although information for IVP embryos in small ruminants is scarce, the convenience of embryo transfer in blastocyst stages instead of morulae is largely accepted. Regarding the maternal component, the recipient female should provide a competent luteal function with sufficient progesterone production as well as a suitable uterine environment for embryo development and placentation. The luteal activity is determined, at least in part, by the treatment applied to synchronize the ovulation. In this sense, traditional hormonal protocols consist of progesterone or progestogen-based treatment usually administrated with intravaginal devices for 10 to 14 days (goats and sheep, respectively) and associated to eCG administration i.m. at device removal, with a luteolytic dose of PGF2alpha in goats. Alternatively, short-term protocols with progestogen treatment during 6-7 days have been used in sheep with similar outcomes than traditional treatments (Menchaca *et al.*; IRAUy, Montevideo, Uruguay; unpublished data). Using this protocol for estrus synchronization, around 90% of estrus and ovulation occurs in average 30 and 60 h from device removal, respectively (Menchaca and Rubianes, 2004). In our routine in sheep, the intravaginal device is inserted on Day 0 and removed in the evening of Day 6 plus PGF2alpha and 300 IU eCG, and marker vasectomized males are introduced into the flock from 24 to 48 h after device removal. Fixed time embryo transfer (FTET) is performed on Day 15 (i.e. 8.5 days after device removal and around 7 days after the onset of estrus) to transfer day 6 blastocysts (i.e. 7 days after follicular aspiration). FTET is carried out only in painted females (i.e. those that were mounted by the males) and the embryos are transferred into the uterine horn ipsilateral to the corpus luteum, assessed by laparoscopy. Expected pregnancy rate is approximately



50-60% when FTET is conducted with fresh embryos. Alternatively, FTET may be performed into the oviduct on Day 10, i.e. 3.5 days after device removal and one day after *in vitro* insemination and ovulation.

The recommended number of IVP embryos to be transferred per recipient is 1 or 2 embryos when FTET is performed with blastocysts, depending on the species, breed and productive system. In our experience, when transferring two IVP embryos around 30% of pregnant ewes get pregnant with twins. In a recent trial in which FTET was conducted with 240 IVP embryos in Merino recipients (a breed having around 1.1 of prolificity), pregnancy rate was about 10% greater when two embryos were transferred ($P < 0.05$). However, birth weight was 20% lower in twins ($P < 0.05$) and lamb survival rate after birth was much greater with single pregnancies (about 30%; $P < 0.05$) (Menchaca *et al.*; IRAUy, Montevideo, Uruguay; unpublished results). For this reason, under typical rangeland conditions as in Uruguay and Argentina, the objective should be to achieve one born lamb per ewe, which ensure a suitable birth weight and lamb survival rate.

Are we ready to skip the fresh embryo transfer?

One of the greatest difficulties of *in vitro* embryo technology in livestock is the low cryotolerance of the produced embryos in comparison to *in vivo* derived (IVD) embryos. For this reason, usually IVEP programs are conducted with fresh embryos, which in large-scale programs with embryos produced every week during long periods of time, require a large and continuous number of ready-to-use recipients. In this context, embryo cryopreservation deserve to be considered.

Conventional slow freezing methods were developed for IVD embryos, and in fact, they are widely applied in sheep and goats in commercial MOET programs. In general, this conventional method results in very low survival rates when applied to IVP embryos (Massip, 2001). *In vitro* produced embryos have some intrinsic differences with IVD embryos that negatively affect cryotolerance, associated with excessive accumulation of lipids, altered metabolism, changes in structural and physic characteristic, among others (Seidel, 2006). For this reason, vitrification methods have been further studied in parallel with the development of IVEP technology in different species, applied in livestock and endangered species, but also demanded in human assisted reproduction for oocyte and embryo cryopreservation. Several studies have been conducted to test different vitrification methods for small ruminants embryos (Traldi *et al.*, 1999; Dattena *et al.*, 2000; Baril *et al.*, 2001; Martínez *et al.*, 2006; Gibbons *et al.*, 2011; Morato *et al.*, 2011). In general, the effectiveness of vitrification technique depends on several factors as the stage of embryo development, embryo origin (*in vivo* or *in vitro*), volume and cooling rate, cryoprotectant media, and the species, among others (Arav, 2014).

During the last years, novel concepts related to vitrification have supported the development of

minimum volume methods, reducing the volume of cryoprotectants and increasing the cooling and warming rates (Yavin and Arav, 2007). We have conducted a series of experiments with ovine embryos to improve survival rate after cryopreservation by using vitrification with minimum volume methods (Cryotop and Spatula MVD). These two vitrification methods have been previously reported for human (Kuwayama, 2007) and mice embryos (Tsang and Chow, 2009), respectively. In a recent study, we found that ovine IVP embryos vitrified with Cryotop and Spatula MVD showed acceptable *in vitro* survival and development rate (dos Santos Neto *et al.*, 2015). In a more recent study (dos Santos Neto *et al.*; IRAUy, Montevideo, Uruguay; unpublished results), we transferred 437 embryos to compare *in vivo* vs. *in vitro* sheep embryos subjected to vitrification by Cryotop or Spatula MVD methods, or to conventional freezing. As expected, regardless the cryopreservation method, pregnancy establishment was greater with IVD than with IVP embryos (pregnancy rate 68.8 vs. 22.3%, $P < 0.05$). Interestingly, recipient females receiving IVD or IVP embryos resulted in greater pregnancy rate ($P < 0.05$) when vitrification was performed by Cryotop method (77.8 and 55.1%) than Spatula MVD (59.3 and 18.4%) or conventional freezing methods (64.9 and 11.1%, respectively). Thus, in these conditions, the Cryotop method reached >50% of pregnant females transferred with IVP embryos. Pregnancy losses from 30 days of gestation to delivery tended to be greater for IVP than IVD embryos (14.9 vs. 5.8%; $P = 0.08$), with no effect of the cryopreservation method (dos Santos Neto *et al.*; IRAUy, Montevideo, Uruguay; unpublished data). Therefore, minimum volume vitrification methods seems to be interesting for future implementation in IVEP programs in sheep. However, some refinements are still necessary in order to have an easy and robust method to be applied on the field, and thus, finally take advantage of the benefits of cryopreservation.

Concluding remarks

The cumulative work achieved during several years by the contribution of many researchers, briefly summarized in this review, have transformed the IVEP in a supporting tool for livestock, genetic engineering and research. However, some limitations needs to be solved in order to improve the outcomes and simplify the technique. Oocyte competence is variable and should be improved, mainly by controlling follicular wave dynamics to offer more healthy oocytes for LOPU. *In vitro* COCs maturation, fertilization and embryo culture is a well-standardized procedure, but still requires some fine-tuning since most of the embryos die during the process before hatching. Embryo transfer and recipients management may be improved by FTET and standardized protocols for ovarian synchronization, as proposed in this review. In addition, fresh embryo transfer is a limiting factor for international exchange of genetic material and requires continuous availability of recipients. In this sense, new minimum volume methods for vitrification seems to be



attractive allowing acceptable pregnancy rates in our system. In summary, although some challenges are still present in IVEP in sheep and goats, substantial improvements have been achieved during the last years and this technology is available to be applied on the field.

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