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Practical aspects for implementing *in vitro* embryo production and cloning programs in sheep and goats

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Abstract

In vitro embryo production has the potential to produce more offspring from genetically valuable animals than standard MOET as it is capable of avoiding most of the causes of failure in MOET (poor response to superovulation, poor fertilization and premature luteolysis). It also allows repeating collection in the donor animals more often and more times in their reproductive life. However, consistent results are not easy to obtain when conducting large scale programs, mainly due to variability associated with the *in vitro* fertilization results. Cloning by somatic cell nuclear transfer has been used by a few groups to successfully produce genetic copies of individuals of high genetic merit, but remains to be a very inefficient reproduction technology even in the hands of those that have been successful in producing multiple live clones. This review provides a collection of tips and points to consider when planning *in vitro* embryo production and cloning programs in sheep and goats.

Keywords: cloning, hyaluronic acid, IVEP, LOPU.

Introduction

In vitro embryo production (IVEP) has been identified as a reproductive technology with the potential to produce more offspring from genetically valuable animals than standard MOET (Baldassarre and Karatzas, 2004; Cognie *et al.*, 2004). In short, the technology can be repeated in the same animal more often and more times in the reproductive life of a female (this is especially true in sheep where embryo collection is surgical); is more reliable/predictable in its results, and it can be conducted in categories that are not eligible for MOET such as prepubertal (Armstrong *et al.*, 1997; Baldassarre *et al.*, 2004) and older females (Baldassarre *et al.*, 2007). In the last two decades, multiple publications have reported the successful production of live offspring (sheep & goat) using this advanced reproductive technology, however, the technique continues to be far away from massive implementation in livestock improvement programs. Some of the limitations for that growth will be discussed later in this manuscript, together with practical tips from 20 yr of practicing the art.

Somatic cell nuclear transfer (SCNT) has resulted in the production of live offspring in both sheep and goats (Wilmut *et al.*, 1997; Baguisi *et al.*, 1999; Keefer *et al.*, 2001; Blash *et al.*, 2012). The main interest of SCNT remains to be its utility for maximizing efficiency in the production of transgenic founder animals, as transgene integration can be conducted by *in vitro* transfection of somatic cells (nuclear donor) and fully characterized in terms of integration events prior to use for reconstructing embryos by SCNT and eventually generating live offspring. Today, the application of transgenic animal technologies with greatest future prospects seems to be the production of animal models for the study of human diseases. As per applications associated with livestock improvement, SCNT in combination with transgenic technologies has been suggested and/or utilized in the production of animals with disease resistance and improved production (Wheeler, 2007). However, it is unclear if and when these applications will gain regulatory approval for marketing, as they face very significant opposition from different consumer and ethics groups. Nonetheless, SCNT is a valuable tool for making genetic copies of elite animals of extreme genetic value, and this manuscript will discuss some of the points to consider when planning and executing a SCNT program in sheep and goat.

The objective of this paper is to provide a step-by-step review of these reproductive technologies, with more focus on IVEP as it has greater interest and possibilities for commercial application, but with reference to some of the key points in SCNT as well.

Laparoscopic ovum pick-up (LOPU)

Whether we are collecting oocytes from valuable donors as part of an IVEP program, or we are collecting oocytes from standard goats to be used as recipient cytoplasts in a SCNT program, it all starts here. This is a very important step as it will have a great impact on performance in the steps that follow; collecting a large number of good quality oocytes is the first key to success.

Donor selection

In an IVEP program, donors are typically selected because of their genetic merit, i.e. similarly to what happens in a MOET program, only the best females in the herd should be selected as donors. However, it is often the case that donors may get

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selected into the program because they are not eligible for MOET. For example, valuable females that have repeatedly resulted in the recovery of unfertilized and degenerative ova only; or females that previously resulted in premature luteolysis when superovulated for embryo recovery. Donors may also be selected because they are too young to breed (prepubertal) and a distinction needs to be made here because this is probably one of the most attractive applications of IVEP, the production of progeny from prepubertal animals which has been discussed in extent in previous publications (Armstrong *et al.*, 1997; Baldassarre and Karatzas, 2004; Baldassarre *et al.*, 2004). Last but not least, LOPU and IVEP can be practiced with success in females of high genetic value that have become too old to respond to MOET, thereby extending their reproductive life as described previously (Baldassarre *et al.*, 2007).

In the case of oocyte donors for SCNT, some people may resort to starting from slaughterhouse ovaries if available in their work area, but we find that the oocyte quality from LOPU-sourced oocytes is much better. In farms where embryo work is conducted regularly it is customary to have a herd of recipients that can be of any breed (usually not purebreds) as long as they are healthy reproductive and otherwise. These animals can be used as oocyte donors in a SCNT program, especially if they have not gone through multiple embryo transfer surgeries before, thereby avoiding the presence of surgical sequels (adhesions) that may challenge the access to the ovaries under laparoscopic observation.

In both cases we want the donors to be in good condition (3-4 using the standard 1-5 body condition scoring scale), and it is advisable to improve their diet starting one month prior to LOPU so we can have a "nutritional flushing" helping with response to hormonal treatments.

Donor treatment

In order to maximize the number and quality of oocytes collected per donor, the females need to be synchronized and hormonally-primed in preparation for LOPU. Estrus synchronization is conducted by means of progesterone or progestagen-containing intravaginal devices (e.g. CIDR; sponges) that are applied for 9-11 days, together with a luteolytic dose of prostaglandin or analog (e.g. cloprostenol) at the time of initiation of gonadotropin treatment. Several treatments have been proposed for stimulation of follicular growth prior to LOPU, the two most popular been a Multiple FSH injection regime and the so-called Oneshot regime (Baldassarre and Karatzas, 2004). In the Multiple FSH regime, three FSH injections of 1.5, 1.5 and 1 ml totalling 80 mg of FSH (Folltropin, Bioniche Animal Health, Canada) are administered at 12 h intervals starting 36 h prior to LOPU. In the Oneshot treatment, 4 ml of FSH together with 300 IU of eCG are administered simultaneously (in different syringes) 36 h prior to LOPU. Work reported previously has shown no significant differences between these two treatments, making the Oneshot treatment more appealing due to its simplicity (Baldassarre *et al.*, 1996, 2002). Based on the experience of our group after thousands of LOPUs practiced in both species, scheduling of donor groups is based on the expectation that oocyte recovery will be at an average of ~10 oocytes per ewe and/or 14 oocytes per doe.

In a recent study, we looked at an alternative Oneshot-FSH protocol without co-injecting eCG (Baldassarre *et al.*, 2011). The designed treatment used 10 ml of a 0.5% hyaluronic acid solution (MAP-5, Bioniche Animal Health) for recomposing the lyophilized FSH inside the Folltropin vial. The hyaluronate acts as a slow releasing factor (SRF) making FSH pass into circulation in a slow manner, and we have hypothesized that this would allow for similar ovarian stimulation results than with a single injection. Another desired advantage was the avoidance of eCG in the protocol, not only for simplification and economic reasons, but mainly because of the immunogenic nature of the hormone (Roy *et al.*, 1999; Drion *et al.*, 2001). Results proved the working hypothesis to be correct as the number of follicles aspirated (17.8 *vs.* 17.9; $P > 0.05$, ANOVA) and oocytes recovered (13.7 *vs.* 14.0; $P > 0.05$, ANOVA) were not different between Control and FSH-SRF goats. Similarly, the number of follicles aspirated (12.6 *vs.* 12.4; $P > 0.05$, ANOVA) and oocytes recovered (10.9 *vs.* 10.8; $P > 0.05$, ANOVA) were not different between Control and FSH-SRF sheep (Baldassarre *et al.*, unpublished data).

Follicular aspiration

LOPU must be conducted under general anesthesia and this requires the goats to be deprived from food (24 h) and water (12 h) prior to the initiation of the procedure. Our preferred method of anesthesia consists of induction with ketamine (5 mg/kg) and diazepam (0.35 mg/kg), followed by inhalation anesthesia with isofluorane. The laparoscopy set of choice consists of a 5 mm, 0° angle telescope, two 5.5 mm trocar/cannula sets (one for the laparoscope and one for the forceps), one 3.5 mm trocar/cannula set for the aspiration pipette; a 5 mm atraumatic grasping forceps; a fiber optic cable and a light source (at least 150W halogen lamp; although the newer xenon lamps are much more expensive but also much better). 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 is handmade by us by fixing a 20 G short bevel needle to transparent acrylic tubing that has a 3 mm external diameter and 1 mm internal diameter. The details of needle size and

placement are important. A smaller needle diameter (e.g. 21-22 G) will likely promote denudation of oocytes as they enter the needle; while bigger needles (e.g. 18 G) will likely do more damage to the ovary and result in more bleeding. Another important aspect is needle length, in relation to how much it flushes from the tip of the acrylic tubing. This should be 5 mm long, again longer needles resulting in higher incidence of damaging the follicle floor and blood in the collection tube. Contrary to what happens in cattle where puncturing the ovarian stroma with the aspiration needle is almost inevitable, if one is careful and punctures the follicle with the right needle in the right angle, the presence of blood in the collection tube is quite avoidable and this facilitates greatly the process of searching and finding the oocytes.

With the goat under general anesthesia and lying securely on a laparoscopy table, while looking through the laparoscope, 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. The fact that the tubing is transparent allows visualizing the column of follicular fluid as it ascends through the tubing, which gives great confidence that the process is going well and all systems are functional. For a trained team like ours, on average it takes about 15-20 min per donor (including preparation). At the end of the procedure donors are given a preventive dose of antibiotics and a non-steroid analgesic prior to placing in the recovery pens. They are returned to their regular pens/paddocks the following day.

In vitro embryo production

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma Chemical Co., St. Louis, MO, USA.

In vitro maturation (IVM)

In vitro maturation is regularly performed in 50 µl drops of maturation medium under mineral oil. The maturation medium consists of M199 supplemented with bLH (0.02 U/ml; Sioux Biochemicals, USA), bFSH (0.02 U/ml; Sioux Biochemicals, USA), 17β-estradiol (1 µg/ml), sodium pyruvate (0.2 mM), kanamycin (50 µg/ml) and 10% heat-inactivated fetal bovine serum (sheep). For goats we use the exact same medium with the only exception that serum is replaced with 10% heat-inactivated estrus goat serum which promotes better nuclear maturation and acquisition of meiosis competence (Palacios *et al.*, 1998). *In vitro* maturation is performed at 38.5°C in humidified atmosphere with 5% CO_2 in air for 24 h.

In vitro fertilization (IVF)

Following IVM, expanded cumulus cells are partially removed from matured COCs by repeated pipetting. Oocytes are washed in fertilization medium and transferred to 40 µl drops of fertilization medium under mineral oil. In goats, fertilization medium consisted of TALP medium (Parrish *et al*., 1986) supplemented with 10% heat-inactivated estrus goat serum, while in sheep we use mSOF medium supplemented with 10% heat-inactivated estrus sheep serum.

In both species, but perhaps more critically in the goat, the IVF step is the bottleneck of the *in vitro* embryo production process. Three elements play a very important role in determining the outcome of an IVF experiment: semen source, serum source and if required, the use of chemicals for capacitation. Finding the right balance between these three components of IVF success will allow avoiding low fertilization rates and/or high polyspermy rates, which are the two main sources of failure. As per semen source, our preferred source is frozen-thawed, especially from batches that have been used previously to optimized IVF conditions have been established. In general, the semen dose will be situated in the range of 50,000 to 150,000 sperm per drop in goats (representing a concentration of 1 to 3 x 10^6 live sperm/drop) while in sheep the range is 50,000 to 100,000 sperm per drop. When frozen semen is not available for the male of interest but the male is available for semen collection, we can conduct the fertilization using fresh semen pre-incubated with IVF medium containing capacitating agents (e.g. heparin 10 µg/ml, 30 min). Alternatively, semen may be collected the day before IVF and left in the refrigerator for 15-20 h after centrifugation to eliminate seminal plasma and re-suspension in milk. While there is no universal recipe that will fit all males and semen samples, overnight refrigerated semen usually is capacitated and readily used in IVF without the need for chemical agents. Estrus serum (sheep and goat) must be inactivated at 56°C, 30 min and tested in IVF replicates prior to use. Once again, we have found that there is no universal recipe, and different serum batches may not work with certain males or may require to be used in a concentration greater than 10% to exert its capacitation functions. Fresh and refrigerated semen with motility ≥70% can be washed by simple centrifugation at 1200 rpm for 10 min prior to re-suspension in IVF medium for use. However, frozen semen needs to be washed and enriched in live sperm by means of a 45:90% Percoll gradient. For that matter, the content of one semen straw is overlaid on top of a Percoll gradient prepared by adding 2 ml of 45% Percoll on top of 2 ml of 90% Percoll inside a 15 ml Falcon tube, and centrifuged a 1200 rpm for 30 min. The pellet is then re-suspended in 4 ml of IVF-medium and centrifuged for 10 min at 1200 rpm. The washed pellet is finally subjected to sperm count using a hemocytometer chamber and diluted with IVF-medium to meet the desired concentration (i.e. 10X of desired concentration in the

IVF drop). After insemination, the IVF drops are incubated at 38.5°C in a humidified atmosphere incubator with 5% CO_2 in air for 15 to 20 h.

In vitro culture (IVC)

In both species, IVC is conducted in drops of mSOF medium under mineral oil, at 38.5°C in a humidified atmosphere incubator with 5% O_2, 5% CO_2 and 90% N_2. The mSOF is the synthetic oviduct fluid medium as originally reported (Tervit *et al.*, 1972), supplemented with 2% essential (BME) and 1% non-essential (MEM) aminoacids and 8 mg/ml fatty acid free bSA.

A matter of debate is whether to culture the embryos for shorter or longer period prior to transfer to recipients (Cox and Alfaro, 2007). Those who advocate for longer culture period will argue that it allows selecting the best suited embryos for transfer thereby needing fewer recipients. If the objective is to produce embryos for freezing/vitrification, there is no doubt that this is the right path since good cryopreservation rates are only achieved with compact morula/blastocyst-staged embryos. However, in the big scheme of things, in large programs and looking at birth rates as main success indicator, we find that more offspring are probably born when Mother Nature is used as incubator.

SCNT embryo production

The procedures for cell preparation, oocyte enucleation, cell injection, fusion and activation for the production of goat embryos reconstructed by SCNT have been described in detail elsewhere (Keefer *et al.*, 2001; Baldassarre *et al.*, 2003). This program conducted with my former group in Canada during 1998 to 2004 resulted in 259 cloned goats born of which 203 lived to become adults. In more recent times, similar protocols with a few improvement/modifications were used for the successful production of cloned goats and sheep with my group in Argentina (Bordignon *et al.*, 2011; Colato *et al.*, 2011). From that experience, the following are points to consider when planning a SCNT program in sheep and goats:

Cell line-related factors

Cell line has been by far the most significant determinant of success in our cloning programs. Without changing anything in the standard operating procedures, pregnancy rates and normal birth rates have varied between 0-80 and 0-70% respectively for different cell lines. Unfortunately, for the moment there is no *in vitro* test that would allow selecting cell lines based on their potential to develop following nuclear transfer. In consequence, the best option is to produce multiple cell lines from animals of interest and to use always more than one cell line when conducting a program. The cell types we have been successful with include fetal fibroblasts, skin fibroblasts and cumulus-granulosa cells. None of them seem to be more successful than the others. Different groups have used different method to induce quiescence in cells in preparation for SCNT. For the work we conducted in Canada, cells were induced into quiescence by using a combination of culture to confluency and serum starvation (0.5% serum in culture), while in the work we conducted in Argentina quiescence was induced by contact inhibition only (i.e. confluency only).

Enucleation

The procedure used by our group in Canada involved staining the oocytes with DNA-specific stain H33342 and a very brief exposure to UV light to visualize the position of the MII chromosomes and remove them by aspiration with a microinjection needle. UV light exposure was controlled by a shutter to ensure it was very short (few seconds). More recently, an improvement has been introduced to this process by incubating the oocytes after IVM for 1 h in medium containing demecolcine which allows visualization of the protrusion made by the metaphase plate usually besides de first polar body. This prevents the need for exposing the oocytes to UV light which has the potential to be harmful to the oocytes.

Fusion and activation

The best recommendation for fusion is to find the optimized parameters for your machine, your fusion chamber and your work conditions. It seems like there is no such thing as a "one fits all" protocol when it comes to fusion. As a reference, most recently we have been using a single DC pulse of 1.6 kV/cm for 70 µs with a BTX ECM 2001 fusion machine in a 0.5 mm gap chamber. The reconstructed embryos were then activated using ionomycin (5 µM/5 min) followed by cycloheximide (10 µg/ml) and cytochalasin B (7.5 µg/ml) for 4-5 h and then transferred into culture medium (mSOF) until transfer.

Improving reprogramming

Multiple cases of development failure in SCNT embryos and newborns have been associated with abnormal epigenomes resulting from inappropriate reprogramming. Conditioning of the media in which embryos are cultured after reconstruction has potential for improving the outcome. For example, increased histone acetylation by exposure to inhibitors of deacetylase enzymes has been reported to improve development of embryos produced by SCNT. In a recent study, we showed that improvements in the production of cloned sheep were possible by using deacetylase inhibitor Scriptaid in the culture media

where reconstructed embryos were cultured after activation and prior to transfer to recipients (Bordignon *et al.*, 2011).

Recipient management

This may seem as the least scientifically challenging aspect of the whole process, but is one that should not be overlooked in order to maximize the efficiencies of IVEP and cloning. Multiple factors need to be taken into consideration.

Recipient selection

Only females with good body condition (3-4) should be used and their diet should be improved in the 2-4 weeks prior to use in order to maximize the response to estrus synchronization treatment and decrease the incidence of premature luteolysis. The use of bigger breeds of goats/sheep and females that have delivered at least once is advisable to prevent issues at the time of parturition. Also, given the fact that transferring multiple embryos per recipient is standard practice in both IVEP and cloning programs, recipients should be of size and breed capable of raising twins and, occasionally triplets.

Recipient synchronization

Recipients must be synchronized to be in heat around the time of IVF (IVEP embryos) or the time of micromanipulation (SCNT embryos). Deviations from this ideal synchrony are more compatible when the embryo is more advanced than the recipient uterus, e.g. it is more acceptable to use a recipient that was in heat the morning after embryo reconstruction than during the afternoon of the day before embryo reconstruction. The rationale behind this concept is that IVEP and SCNT embryos may often be delayed in producing interferon-tau to stop the luteolytic process in normal cycling females. Estrus synchronization is achieved by means of intravaginal devices containing progesterone/progestagen (CIDR, sponges) inserted for 9-11 days in combination with 300-500 IU of eCG and a luteolytic injection of 125 µg cloprostenol. Increased synchrony of ovulation and lower incidence of unovulated follicles (often inductors of premature luteolysis) can be achieved by injecting GnRH (50-100 µg) or hCG (500 UI) 36 h after sponge/CIDR removal (Saharrea *et al.*, 1998; Pierson *et al.*, 2003).

Embryo transfer surgery

Depending on the *in vitro* culture strategy chosen, IVEP and cloned embryos are transferred into the oviduct (short IVC) or uterus (long IVC) of recipient females. Both procedures require a midventral laparotomy under general anesthesia in order to exteriorize the reproductive tract (oviduct transfers) or just the uterine horn (uterine transfer). Prior to transfer it is best to explore the ovaries by laparoscopy in order to ensure the recipient is eligible for embryo transfer, i.e. has at least one recent ovulation/corpus hemorrhagicum and no uterine contents (oviduct transfers); or at least one morphologically normal corpus luteum and no uterine contents (uterine transfer). Embryos are prepared for transfer by loading into a Tomcat® catheter (Sovereign, Canada). In the case of oviduct transfers, the catheter is introduced into the oviduct through the fimbria. For uterine transfers, the wall of the uterine horn is perforated in the tubal third of the horn with an 18 G needle, and the Tomcat containing the embryos is introduced through this perforation into the lumen of the uterus where embryos are deposited. The recommended number of embryos transferred per recipient is 1-2 for late IVEP embryos (blastocyst stage); 3-5 for early IVEP embryos (2-4 cell stage) and 8-12 for SCNT embryos transferred within 24 h from fusion (1-2 cell stage).

After transfer care

Preventive antibiotic (e.g. Tetracycline L.A.) and non-steroid analgesic should be administered before removing the animal from the surgery table. Recipients should be kept in a clean pen under close monitoring for 2-3 days before returning to their regular pens/paddocks in order to avoid intense exercise and/or accumulation of dirt in the surgical wound for the first few days. Ultrasound scans for pregnancy detection are conducted at 4 and 8 weeks of pregnancy. Most pregnancies losses occur between first and second ultrasound scans (IVEP and cloned embryos). In the case of cloned embryos there is also a significant potential for life threatening situations at the time of birth; preparedness for special needs (oxygen, infrared lamp or incubator, colostrum bank, etc.) are key to survival of newborns with compromised conditions. For intensive breeding operations, it is advisable to have a scheduled birthing protocol where recipients are induced into parturition, allowing the newborns to be delivered on days of the week and times of the day that are compatible with maximum staff availability. The recommended protocol for inducing parturition includes estradiol cypionate (1 mg total); cloprostenol (250 µg total) and dexamethasone (3 x 4 mg at 12 h interval). Parturition occurs ~30-36 h after prostaglandin injection in goats and 48-72 h after prostaglandin + first dexamethasone in sheep.

Conclusions

IVEP and SCNT have been integrated recently into the toolbox of small ruminant practitioners in countries where these livestock productions are well developed. Possibly the most limiting factor for

implementation of large scale programs is the need for having a dedicated laboratory with all the necessary equipment to conduct these procedures. Next as a limiting factor is the need for consistent results (IVEP) and higher efficiencies in the production of live offspring (SCNT). While there are multiple sources of inconsistency and low success that are inherent to each technology, this manuscript has provided a volume of tips and points to consider for increasing success rates when planning IVEP and SCNT.

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