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In vivo and *in vitro* embryo production in goats☆

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ABSTRACT

Assisted reproductive technologies (ART) such as artificial insemination (AI) and multiple ovulation and embryo transfer (MOET) have been used to increase reproductive efficiency and accelerate genetic gain. The principal limitations of MOET are due to variable female response to hormonal treatment, fertilization failures and premature regression of *Corpora luteum*. The *in vitro* production (IVP) of embryos offers the possibility of overcoming MOET limitations. The method of IVP of embryos involves three main steps: *in vitro* maturation of oocytes (IVM), *in vitro* fertilization of oocytes (IVF) with capacitated sperm and *in vitro* culture (IVC) of embryos up to blastocyst stage. Recovering oocytes from live selected females by laparoscopic ovum pick-up (LOPU) and breeding prepubertal females by juvenile *in vitro* embryo technology (JIVET) will allow a greater production of valuable goats. Also, IVP of goat embryos will provide an excellent source of embryos for basic research on development biology and for commercial applications of transgenic and cloning technologies. Different protocols of IVP of embryos have been used in goats. However oocyte quality is the main factor for embryos reaching blastocyst stage from IVM/IVF/IVC oocytes. One of the principal determinant factors in the results of blastocyst development is the age of the oocyte donor females. In goats, oocytes from prepubertal and adult females do not show differences in *in vitro* maturation and *in vitro* fertilization; however the percentage of oocytes reaching blastocyst stage ranges from 12 to 36% with oocytes from prepubertal and adult goats, respectively.

1. Introduction

Two exciting developments in goat reproduction are *in vivo* embryo production or multiovulation embryo transfer (MOET) and *in vitro* embryo production (IVEP), the technologies for altering or manipulating genetic material to improve the genetic structure of animals. The reproductive technology most commonly used to accelerate genetic gain has been artificial insemination (AI). However, although MOET cannot replace AI as a routine reproductive technology, it can be applied to allow extra genetic gain through the production of embryos obtained from selected females and males.

In vitro embryo production technology also presents the following advantages: (i) a significant increase of embryos from high genetic value females because oocytes can be recovered from prepubertal, pregnant and even dead or slaughtered goats, (ii) provides an excellent source of low cost embryos for basic research, embryo biotechnology studies (nuclear transfer, transgenesis, embryo sexing and stem cells) and all kinds of embryo research which need high number of embryos for manipulation and (iii) used as a strategy for the rescue of some endangered animal species by interspecific embryo transfer. Moreover, embryo cryopreservation allows the movement and marketing of goat germplasm providing safe worldwide movement of livestock.

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2. *In vivo* embryo production

In vivo embryo production in goats has been studied for years; however the results are not conclusive. The variability of the hormonal treatment, fertilization failure and the premature regression of *Corpora luteum* still needs to be improved (Cognie et al., 2003).

Traditional superstimulatory protocols consist of a prolonged progestagen priming (12–18 days), with FSH administered twice daily for 3–4 days, beginning between 1 and 3 days before the end of the progestagen treatment. On average 8–16 ovulations are generated, although individual variability is immense (Holtz, 2005). Several attempts have been made in order to reduce this labor-consuming protocol. Thus, Pintado et al. (1998) did not find differences by substituting the last three of six FSH-injections by a single dose of 200 IU eCG. When fixed-time insemination is intended ovulation has to be timed. This may be accomplished by injection of LH, human chorionic gonadotropin (hCG) or a GnRH-agonist. Baril et al. (1996) concluded that, in superovulated goats, GnRH is not an efficient means of synchronizing ovulation unless preceded by treatment with a GnRH-antagonist. The antagonist temporarily suppresses FSH- and LH-release and, thus prevents the emergence of a dominant follicle. Some strategies have focused on starting the superovulatory treatment at wave emergence (in the absence of a dominant follicle). Rubianes and Menchaca (2003) recommend initiation of superovulatory treatment concomitant with the first follicular wave emerging after ovulation (day 0). Soon after ovulation, wave 1 emerged and there was a homogeneous cohort of growing small follicles. At day 0, FSH treatment is initiated with six decreasing doses given twice daily. Two half-doses of PGF2α were given concurrent with the fifth and sixth FSH treatments. To synchronize the ovulation GnRH analogue was injected 24 h after the first PGF2α treatment. Results of this protocol showed higher ovulation rates and embryo yield than the traditional protocol (Menchaca et al., 2007).

To collect embryos by laparotomy, uterine horns must be flushed with medium to retrieve the embryos at 6–8 days after insemination. This procedure allows 2–3 collections per goat, because post-operative adhesions are a frequent sequel, limiting the number of possible collections. Laparoscopic embryo collection is less invasive and allows 7 collections (Baril et al., 1996). Collection via cervix, using a rigid catheter, is described by Sohnrey and Holtz (2000).

In a successful goat MOET program an average of 6–8 transferable embryos per donor can be produced, however, many factors (including breed, age and nutrition) contribute to the high variability of transferable embryos (range from 0 to 30 per donor) with 25–50% of the donors failing to produce embryos due to fertilization failure and early regression of *corpora luteum* (Baril et al., 1993; Cognie, 1999; Cognie et al., 2003).

3. *In vitro* embryo production

The method of IVP of embryos involves three mains steps: *in vitro* maturation of oocytes (IVM), *in vitro* fertilization of oocytes (IVF) with capacitated sperm and *in vitro* culture (IVC) of embryos until blastocyst stage that can be transferred to recipient females or cryopreserved for future use.

3.1. Oocyte collection

Collection of good-quality oocytes is the first step for *in vitro* embryo production:

- *Oocytes recovered from slaughtered animals*: Oocytes are liberated from the follicles by aspiration, slicing or follicle dissection. In adult goat ovaries, conventionally, oocytes are recovered by follicle aspiration selecting follicles bigger than 3 mm diameter. From prepubertal goat ovaries, slicing the ovary allows collection of more oocytes per ovary than by follicle aspiration (6.05 and 1.27), but the morphological quality is lower (Martino et al., 1994a).
- *Oocytes recovered from live goats*: The techniques used are the aspiration of follicles after surgical exposure of the ovary by laparotomy or through laparoscopic ovum pick-up (LOPU). In order to recover high number of oocytes, the donor goats are estrus synchronized and stimulated with several doses of gonadotropins. Baldassarre and Karatzas (2004), using a unique injection of 80 NIH-FSH-P1 and 300 IU of eCG at 36 h prior to LOPU obtained an average of 13.5 oocytes per goat.

Transvaginal ultrasound-guided aspiration (TUGA) technique used in goats has been described by Graff et al. (1999).

3.2. Effect of the age of the goat donors on oocyte quality

Several studies have reported lower embryo competence from oocytes of prepubertal than adult females (reviewed by Armstrong, 2001). Previous experiments also reported a lack of development up to the blastocyst stage in prepubertal goats. A percentage of blastocysts of 10% was obtained by Izquierdo et al. (2002) using oocytes from 2-month-old females obtained in a slaughterhouse and 8% of blastocysts was obtained by Koeman et al. (2003) with oocytes collected by LOPU from 2- to 5-month-old hormonally stimulated goats. Crozet et al. (1995) suggest that only a small proportion of the oocytes recovered from 2 to 3 mm diameter follicles can support embryonic development because the capacity to complete cytoplasmic maturation develops beyond the acquisition of meiotic competence. Thus, they showed that the goat blastocyst production was 6% with oocytes from follicles of 2–3 mm, in adult goats. In prepubertal goats, the number of follicles bigger than 3 mm per ovary is 1.1 (Martino et al., 1994a), which means that follicles larger than 5 mm in diameter are practically non-existent in these females. In prepubertal goat oocytes, the percentage of blastocysts obtained is lower than those obtained from oocytes of adult goats. Thus, Cognie et al. (2003) obtained 36%, Crozet et al. (1995) 26% and Keskintepe et al. (1998) 32% of blastocysts from adult goat oocytes.

Studying the differences between oocytes from prepubertal and adult goats, we have found a lower male

pronucleus formation (Mogas et al., 1997a), a high rate of haploid (Villamediana et al., 2001), polyspermic zygotes (Mogas et al., 1997a) and abnormal distribution of cortical granules (Velilla et al., 2004) and mitochondrial morphology (Velilla et al., 2006) in oocytes from prepubertal females.

3.3. Effect of follicle and oocyte size

Several studies in different species have concluded that oocyte diameter is directly proportional to follicle diameter. Increase in follicle and oocyte diameters improve embryo development (reviewed by Gandolfi et al., 2005). In adult goats, Crozet et al. (1995) found a significant difference in the percentage of blastocysts obtained from oocytes recovered from follicles of 2–3 mm (6%), follicles of 3.1–5 mm (12%), follicles >5 mm (26%) and from ovulated oocytes (41%). In prepubertal goats, most of the oocytes come from 2 to 3 mm diameter follicles. In a recent study, we have tested the relationship between follicle diameter and oocyte competence (Romaguera and Paramio, unpublished data). In this study, in prepubertal goats, the percentage of blastocysts obtained from follicles bigger and smaller than 3 mm was 18.5% and 3.85%, respectively. As was indicated previously, follicular aspiration is difficult in prepubertal goat ovaries. Thus, it is easier to select oocytes liberated by slicing, according to their diameter and cumulus morphology. In our previous laboratory studies oocyte diameter and blastocyst development after IVF concluded that a higher blastocyst rate was obtained in oocytes larger than 135 μm (12.5%) compared to oocytes of 125–135 μm diameter (1.95%) (Anguita et al., 2007). However, using ICSI, we did not find differences between these two oocytes categories, 11.1% and 15.9%, of blastocysts (Jimenez-Macedo et al., 2006). In both studies, oocytes of 110–125 μm diameter were able to develop up to morulae but they did not reach blastocyst stage. Oocytes smaller than 110 μm were unable to cleavage. Comparing prepubertal to adult goat oocytes, recovered by LOPU, Baldassarre and Karatzas (2004) found lower embryo production and pregnancy rates in oocytes from prepubertal than from adult goats.

3.4. In vitro maturation of oocyte

Embryo development is influenced by events occurring during oocyte maturation. For successful IVM, oocytes must undergo synchronically nuclear and cytoplasmic maturation. Immature goat oocytes are conventionally matured in buffered TCM199 supplemented with L-glutamine, pyruvate, hormones (FSH, LH and 17β - estradiol) plus serum.

Maturation media are generally supplemented with 10–20% heat-treated serum. In goats, estrous goat serum (EGS) and estrus sheep serum (ESS) are routinely used by several laboratories (Cognie et al., 2003). Tajik and Esfandabadi (2003) did not find differences between EGS, ESS and fetal calf serum (FCS). In our laboratory, we have tested EGS (at different times of estrus), fetal calf serum (FCS) and steer serum (SS) and did not find any significant differences on maturation and embryo production (Mogas et al., 1997b).

Follicular fluid (FF) from non-atretic and large follicles (>4 mm) has been used as a compound of maturation supplementation with good results (Cognie et al., 2003).

Estrus goat serum and FF need to be tested before being integrated in a protocol of *in vitro* embryo production because both compounds present high chemical variations between samples. Rodriguez-Dorta et al. (2007) in adult goat oocytes used a maturation medium with defined compounds. The IVM used is TCM199 supplemented with 10 mg/ml EGF and 100 μM cysteamine, with good results in embryo development.

The addition of different thiol compounds (cystine, cysteine, cysteamine, glutathione, β-mercaepthanol) to the IVM media improve embryo development, increases intra-cytoplasmic glutathione concentration (GSH) and protects cells from culture oxidative stress. In prepubertal goat oocytes, testing different thiol compounds, cysteamine has been the thiol which significantly increased intra-cytoplasmic GSH and embryo development (Rodriguez-Gonzalez et al., 2003a). Supplementation with 100 μM of cysteamine improved embryo yield in oocytes from adult (Cognie et al., 2003) and prepubertal goats (Urdaneta et al., 2003). Zhou et al. (2008) with denuded oocytes (DOs) of adult goats, restored the GSH level and developmental capacity of DOs with cysteamine and cystine.

3.5. In vitro fertilization

Before fertilization, buck ejaculates need to be prepared to inseminate the oocytes. The first step is to select the most motile and viable spermatozoa from the whole fresh ejaculate or the frozen-thawed sperm. The principal techniques used to select spermatozoa are swim-up and centrifugation in Percoll or Ficoll density gradient. Greater yields of highly motile spermatozoa can be obtained by swim-up, when compared to Ficoll or Percoll density gradient centrifugation, but no differences were observed in terms of oocyte penetration and cleavage rate after IVF with fresh goat semen (Palomo et al., 1999). For frozen-thawed goat semen, conventionally, motile spermatozoa are obtained by centrifugation on a discontinuous Percoll gradient. Once the most viable and motile spermatozoa were selected, sperm capacitation is carried out in media supplemented with heat-inactivated estrus serum (20% with fresh semen, 2% with frozen-thawed semen). Cognie et al. (2003) report capacitation of frozen-thawed sperm using 10% (v/v) estrus sheep serum in SOF medium and 0.5 μg/ml of heparin during 1 h. In our laboratory, after fresh sperm selection by swim-up, the supernatant is recovered and capacitated in mDM with 50 μg/ml heparin for 45 min.

After oocyte maturation and sperm capacitation, oocytes are transferred to microdrops of modified Tyrode's medium (TALP) as described by Parrish et al. (1986), supplemented with hypotaurine and glutathione. Different fertilization media have been used by different authors: BO (Crozet et al., 1995; Ongeri et al., 2001), SOF (Rho et al., 2001) and TALP-fert medium (Katska-Ksiazkiewicz et al., 2004; Wang et al., 2002).

3.6. Intra-cytoplasmic sperm injection (ICSI)

Intra-cytoplasmic sperm injection has been introduced as an alternative to assisted reproduction technology, especially in humans. A major application of this technique for animal production includes use of genetically important male gametes for procreating wild and domestic animals. Moreover, this technique can be used to extend the sperm vector for transgenic animal production and to use freeze-dried sperm for which spermatozoa motility is not required. The first live kids have been obtained by Wang et al. (2003). In our laboratory, the protocol used by ICSI consists in placing one matured oocyte into a microdrop of 5 μl of injection medium (TCM199) covered with mineral oil. A small volume (1 μl) of sperm suspension is added to another 5 μl drop with a 10% polyvinilpirrolidone (PVP) medium. The injection pipette has an inner diameter of 7–9 μm and the holding pipette measures 20–30 μm. The spermatozoon is expelled into the ooplasm with a minimum volume of medium (<5 pl). Using fresh semen and capacitation with heparin (50 μg/ml), the injected oocytes had to be activated chemically (with ionomycin and 6-DMAP) to start oocyte cleavage. This activation protocol induced a high percentage of parthenogenic embryos. A second protocol was carried out to overcome parthenogenesis. After sperm selection, sperm were capacitated with high concentrations of sperm capacitator compounds (heparin plus ionomycin). Blastocyst yield from ICSI-oocytes of prepubertal goats was 16% (Jimenez-Macedo et al., 2006, 2005). In adult goats, Keskintepe et al. (1997) obtained 18% and Wang et al. (2003) a 32% of blastocysts.

3.7. In vitro embryo culture

After 24 h post-insemination (IVF or ICSI), presumptive zygotes are removed from the fertilization medium and placed in an embryo culture medium. Early goat embryos cultured *in vitro* fail to develop past the 8–16-cell stages in traditional culture media. This block occurred around time of activation of the embryonic genome. Serum and cells are added to the culture to avoid this block.

Embryos can be cocultured with different types of cells. Coculture with granulosa cells (GC) in TCM199 improved embryo development compared to culture media without cells. However, goat oviduct epithelial cells (GOEC) resulted in higher embryo development than GC-coculture (Izquierdo et al., 1999). Yadav et al. (1998) did not find differences between GOEC and buffalo oviductal cells, but both treatments improved the percentage of blastocysts compared to TCM199 without cells. Katska-Ksiazkiewicz et al. (2004) in coculture with GOEC, from oviducts of non-synchronized donors, in B_2 medium reached 37% of blastocysts. Pawshe et al. (1996) reached 40% of blastocysts using TCM199 with GOEC plus EGF, insulin, transferrin and selenite in an aerobic atmosphere. Cells are important in aerobiosis culture conditions (5% CO_2 in air), but they are not as necessary in an anaerobiosis atmosphere (atmosphere of 5% CO_2, 5% O_2 and 90% N_2). Cells are an important source of media contamination and the results are not predictable because of the unknown physiological status of the cells.

In anaerobiosis conditions, different media are used: Sequential medium, G1.2 and G2.2 (Jimenez-Macedo et al., 2005; Koeman et al., 2003; Wang et al., 2003), SOF medium (Anguita et al., 2007; Cognie et al., 2003; Jimenez-Macedo et al., 2006; Keskintepe and Brackett, 1996; Rodriguez-Dorta et al., 2007) and TCM199 medium (Izquierdo et al., 1999). Rodriguez-Dorta et al. (2007), comparing embryo development of adult goat oocytes, concluded that the development of zygotes in SOF medium resulted in higher blastocyst yield than in coculture with GOEC monolayer (28% and 20% of blastocysts, respectively), but after embryo vitrification the percentage of embryos obtained with GOEC significantly improved the rate of pregnancy and survival of embryos giving normal gestation as well as the birth of healthy offspring. In our laboratory the culture medium used is SOF (1 μl/embryo) plus FCS (0.1 μl per embryo) added 24 h after presumptive zygotes were placed in the culture medium.

4. Conclusions

Despite recent progress made in MOET methodologies more research is needed to know the response, in ovulation rate and embryo recovery, to exogenous hormones according to the follicular state of the ovary. Knowledge of the follicular status and its repercussions on molecular characteristics of oocytes will be the major challenge to optimizing both MOET and IVEP methodologies. In recent years IVEP has improved significantly. One of the reasons is the important number of research teams working in this field around the world. However the already dramatic difference in IVEP results between laboratories and within laboratories is mostly due to the unknown oocyte quality. The optimization of IVEP procedures must be consolidated over deep and basic knowledge of the biological material we are using, mostly the oocytes, but also the spermatozoa. This knowledge will be fundamental in improving goat productivity but will also be vital for the production and propagation of transgenic and cloned animals.

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