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In vitro production of embryos in South American camelids☆

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ABSTRACT

Studies in reproductive biotechnology techniques have been minimal in South American camelids (SAC). Complex reproductive characteristics of these species contribute to slow progress. Nevertheless, some techniques, such as *in vitro* fertilization, intracytoplasmic sperm injection and nuclear transfer have been applied and have produced advances in knowledge on embryo environment and *in vitro* conditions necessary for development. Embryo production may have a high impact in both domestic and wild camelids population. Studies addressed to improve *in vitro* embryo production and oocyte collection could be a potential key to develop IVF and embryo production as a routine procedure in camelids.

1. Introduction

Reproductive biotechnology techniques, which include artificial insemination with cooled or frozen-thawed semen, embryo transfer (ET), *in vitro* embryo production (*in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI) and cloning) are widely used in domestic species such as bovines and equines. However, the development of these techniques has been slow in South American Camelids (SAC). Nevertheless, the application of some assisted reproductive technologies, such as synchronization of ovarian follicular development, ovarian superstimulation and ET, have increase in camelids whereas artificial insemination, IVF and ICSI are still in progress (Tibary et al., 2005; Miragaya et al., 2006).

Applying biotechnology such as *in vitro* embryo production offers the possibility of increasing knowledge regarding on embryo or gamete physiology and will be facilitate the development of assisted reproductive technique in these species.

The objective of this review is to document factors affecting *in vitro* embryo production in SAC including all the process involved in this technology such as oocyte collection, *in vitro* oocyte maturation, semen preparation (semen characteristics, separation of spermatozoa from seminal plasma and selection of motile spermatozoa), IVF, ICSI, nuclear transfer and *in vitro* culture of embryos.

2. Oocyte collection

2.1. Oocyte collection from slaughterhouse ovaries

A great number of oocytes can be collected by slaughterhouse's ovaries. However, it is not known if follicles considered as pre-ovulatory were in the growing or the regressing phase, which would affect the quality of the

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oocyte and they also required being *in vitro* matured (IVM). Two methods of oocyte collection have been used in SAC: slicing of ovaries (alpacas: Condori et al., 2010; llamas: Del Campo et al., 1994) and aspiration of ovarian follicles with a needle attached to a syringe (alpacas: Huanca et al., 2009; llamas: Del Campo et al., 1992; Ratto et al., 2005). Slicing has produced the highest yield of oocytes (27 per female), but obtains a heterogeneous population of oocytes from preantral and antral follicles that decreases *in vitro* maturation rate (Del Campo et al., 1994). Aspiration of follicles from 1 to 12 mm resulted in an average of 7 oocytes per female (Del Campo et al., 1992) and 2.3 oocytes per ovary were obtained after aspiration of 3–6 mm follicles (Ratto et al., 2005).

2.2. Oocyte collection from in vivo animals

Obtaining gametes from live animals ensures the oocytes are recovered from follicles in the growing phase. Also, this approach allows producing embryos from genetically superior animals. An ovarian superstimulatory treatment is applied in these animals prior to obtaining the oocytes, because stimulating the growth of multiple follicles allows intensive use of these females. According to Bourke et al. (1995), when implementing ovarian superstimulation, it is necessary to start hormone treatment in the absence of dominant follicles, because when starting the treatment in the presence of a follicle larger than 5 mm, growth of only that follicle is induced (Miragaya et al., 2006).

Oocytes collected by laparotomy have resulted in one of most efficacy technique in SAC. The cumulus oocyte complexes (COC's) can be obtained from over 80% of the follicles that are aspirated (llama: Trasorras et al., 2009; alpaca: Gomez et al., 2002; Ratto et al., 2007) and a relationship exists between the size of the follicle and the stage of maturation of the COC that is recovered (Trasorras et al., 2009). Chaves et al. (2004) recovered a total of 46 oocytes from the 83 follicles aspirated in vicunas (recovery rate: 55.4%). However, this method is invasive and caution must be considered during and after the procedure. An alternative method to the surgery is ovum pick-up using the ultrasound-guided transvaginal. Although there are not very many reports of this technique in llamas, the percentage of COC's recovery varies between 52% (Brogliatti et al., 2000), 74% (Ratto et al., 2002) and 77% (Berland et al., 2011) in superstimulated females. Both follicle aspiration techniques (laparotomy and ultrasound-guided transvaginal aspiration) have the disadvantage of possible bleeding after aspiration and subsequent adherences being formed in the ovarian bursa due to the release of fibrin. There are currently no reports of ultrasound-guided transvaginal follicle aspiration in alpacas; perhaps their small size has hindered the maneuver in these animals.

3. *In vitro* oocyte maturation

In vitro maturation conditions from SAC oocytes are similar to those for ruminants: oocytes are *in vitro* cultured in a maturation medium consisted of TCM-199 supplemented with 10% fetal calf serum (FCS), 0.5 μg/ml FSH, 5 μg/ml LH, 1 μg/ml estradiol-17β and 25 μM pyruvate at 38 °C, with 5% CO_2 in humidity atmosphere (Del Campo et al., 1992; Ratto et al., 1999). Various authors have studied the interval of time necessary to reach the metaphase II (MII) in oocytes collected from slaughterhouse ovaries. The first report of IVM of llama oocytes was published by Del Campo et al. (1992). They have studied different times of oocytes cultured at 0, 18, 24, 36 and 48 h and those having attained MII were considered matured. *In vitro* culture for 36 h resulted in a significantly higher rate of oocytes in MII (62%) compared to the other incubation times. On the other hand, Ratto et al. (2005) reported a higher proportion (78%) of oocytes in MII at the shortest time interval (28 h) compare with Del Campo et al. (1992). Besides, Sansinena et al. (2003) obtained 52% oocytes in MII after 30 h of maturation. In alpacas, Huanca et al. (2009) *in vitro* cultured oocytes for 30, 34 and 38 h and obtained 19%, 43% and 66% maturation rate respectively. Complete maturation involves both nuclear and cytoplasmic maturation. The most commonly used marker for cytoplasmic maturation is evaluation of the movement of the cortical granules toward the periphery of the oocyte. This evaluation can be carried out by confocal laser microscopy using different fluorochromes. Currently there are no reports on evaluation of cytoplasmic maturation of either llama or alpaca oocytes obtained from slaughterhouse ovaries. Nevertheless, the complete maturation process has been studied in oocytes obtained from live animals such as vicunas (Chaves et al., 2004). Vicuna's follicles were surgically aspirated and IVM was carried out in TCM 199 for 27 h at 38 °C with 5% CO_2 and 100% humidity. The percentage of matured oocytes (MII) was evaluated by confocal laser microscopy using propidium iodide (PI) to stain nuclear material and fluorescein marked peanut agglutinin (FICT-PNA) to stain the cortical granules. After incubation, 41% of the oocytes had reached nuclear maturation, with extrusion of the first polar body, and all showed cytoplasmic maturation; although in this study, oocytes were recovered from superstimulated females.

4. *In vivo* oocyte maturation

As it have been seen before, the rate of *in vitro* oocyte maturation in SAC remains variable. In bovines, *in vivo* maturation is more efficient for reaching the blastocyst stage than *in vitro* maturation (van de Leemput et al., 1999; Rizos et al., 2002). As camelids are induced ovulators, *in vivo* oocyte maturation within the follicles could be produced by the induction of an LH surge using exogenous administration of GnRH analogs (Gonadotrophin Releasing Hormone) such as buserelin or directly hCG. Miragaya et al. (2002) collected oocytes by surgical aspiration 22 h after buserelin administration in llama superstimulated with eCG (500 IU) and obtained 66% oocytes in MII. In llamas, superstimulation with eCG is associated with a greater proportion of expanded COC's and COC's in MII, compared to treatment with FSH (Ratto et al., 2005); although in alpacas the same researchers obtained the opposite effect (Ratto et al., 2007). Trasorras et al. (2009) obtained 94% (98/104) COC's in the expanded stage from follicles ≥7 mm in diameter in llamas supestimulated with eCG; and no compact COC's (0/104) were recovered. Buserelin administration was

beneficial for recovering a larger quantity of expanded COC's, which can be used directly in assisted reproductive techniques without needing prior *in vitro* maturation (Trasorras et al., 2009). In vicunas, oocyte nuclear and cytoplasmic maturation was studied in superstimulated females with 750 IU of eCG (Chaves et al., 2004). These authors found that 45% of the oocytes showed nuclear maturation but only 9% also showed cytoplasmic maturation. These results demonstrated the beneficial effect of the superstimulatory treatment despite the absence of the induction of ovulation.

5. Semen preparation

5.1. Semen characteristics

South American camelid semen presents very particular characteristics, such as high structural viscosity (Casaretto et al., in press) and when pipetted, ejaculates form a thread of variable length. Contrary to other species, semen liquefaction can take several hours (24–48 h) or not occur at all (Garnica et al., 1993; Apichela et al., 2007). This characteristic makes semen management very difficult in the laboratory (Tibary and Vaughan, 2006), hindering the separation of spermatozoa from the seminal plasma and the isolation of motile from immotile sperm (Sansinena et al., 2007; Conde et al., 2008; Giuliano et al., 2010). In addition, spermatozoa from these species show oscillatory movements in the ejaculate rather than progressive motility when the ejaculate is not treated (Lichtenwalner et al., 1996; Vaughan et al., 2003; Giuliano et al., 2010). Due to these characteristics of raw semen samples, Del Campo et al. (1994) used epididymal spermatozoa from slaughtered animals to obtain llama embryos, while Sansinena et al. (2007) discarded the use of IVF, working only with ICSI.

Embryos have been produced *in vitro* using spermatozoa from the epididymis (Del Campo et al., 1994; Gomez et al., 2002; Gamarra et al., 2008; Huanca et al., 2009, 2010; Condori et al., 2010; Berland et al., 2011) or from ejaculates (Miragaya et al., 2003; Sansinena et al., 2007; Conde et al., 2008; Trasorras et al., 2010a). The advantages of using epididymal spermatozoa are that these cells are progressively motile and sample management is easier because of the absence of seminal plasma. The main disadvantage is not being able to use this protocol in genetically superior males. On the other hand, ejaculated spermatozoa do not present this last problem but have the disadvantage that most sperm do not present progressive motility and sample management is difficult due to the viscosity of the seminal plasma accompanying the sperm.

If IVF and ICSI are to become acceptable methods for using with males of high genetic value it is mandatory to improve semen collection and preparation techniques, to allow separation and selection of highly motile spermatozoa from seminal plasma.

5.2. Semen viscosity

Different enzymes have been used to improve the rheological characteristics of SAC seminal plasma, with varying results (Bravo et al., 1999, 2000; Poblete et al., 2003; Maxwel et al., 2008). Our laboratory has implemented a protocol using a solution of 1 mg/ml collagenase (Giuliano et al., 2010). The protocol consists of diluting the ejaculate 4:1 in a solution of 1 mg/ml collagenase in TALP medium (Parrish et al., 1986) supplemented with 15 mM Hepes (H-TALP) and 3 mg/ml BSA and incubating at 37 °C for 4 min. Similar results have been obtained following dilution of the ejaculate 8:1 in a solution of 1 mg/ml collagenase in H-TALP-BSA and incubating at 37 °C for 8 min. This protocol has permitted *in vitro* llama embryo production using ejaculated spermatozoa (Conde et al., 2008; Trasorras et al., 2010a).

5.3. Sperm separation

The techniques for semen preparation for *in vitro* fertilization must achieve a high percentage of motile spermatozoa, with normal morphology, free from cell debris and dead spermatozoa. In addition, the sperm separation technique should isolate as many motile spermatozoa as possible and should not cause sperm damage or physiological alteration (Henke and Schill, 2003).

All studies that have been carried out in SAC *in vitro* embryo production report the use of density gradient centrifugation. As can be observed in Table 1, most authors have used discontinuous Percoll gradient centrifugation. As the use of Percoll presents the risk of contamination with endotoxins (Henke and Schill, 2003) washing selected sperm is recommended and this can induce damage to the sperm as well as involving a longer treatment period. For all these reasons, our laboratory has implemented a sperm selection protocol using Androcoll-E^{TM} and has obtained pellets with $10–40 \times 10^6$ sperm/ml, with a 20–60% progressive motility and 20–80% live spermatozoa (Santa Cruz et al., 2010). Added to this, embryos have been obtained by IVF using this sperm selection protocol for the ejaculated semen (Trasorras et al., 2010a).

6. IVF and ICSI

There are few reports published on IVF in SAC. Table 1 shows a summary of the IVF and ICSI techniques applied in SAC published to date. The first embryos produced by IVF were reported by Del Campo et al. in 1994. In the same study, oocytes were collected from slaughterhouse ovaries and IVF was done using epididymal spermatozoa. Embryos were co-culture with llama oviduct cells. Eleven out of the 234 zygotes (4.7%) cultured developed to the hatched blastocyst stage. Gomez et al. (2002) reported the first production of alpaca-llama crossbreed embryos after heterologous IVF using slaughterhouse alpaca oocytes and llama epididymal spermatozoa in co-culture with bovine oviduct cells. They used a small number of oocytes for fertilization and subsequent *in vitro* culture ($n = 5$); after 6 days all had reached the morula stage, but none continued development. They repeated the production of alpaca-llama crossbreed embryos by IVF but the oocytes were recovered from superstimulated females and obtained same embryo stages after 7 day of culture (Ratto et al., 2007). Gamarra et al. (2008) produced hatched alpaca blastocysts

Table 1
In vitro fertilization and ICSI techniques applied in SAC.

Species	Oocyte recovery	Spermatozoa	Density gradient	Progressive Mot. Sperm.(%)	Technique applied	Culture media	Days of culture	Embryo stage reached	Authors/year
Llama	Slaughterhouse	Epididymal[a]	Percoll (90% and 45%)	Not reported	IVF	Oviduct cells	9	Hatched blastocysts	Del Campo et al. (1994)
Alpaca/Llama	Slaughterhouse	Llama epididymal[a]	Percoll	Not reported	IVF	Bovine oviduct cells	6	Morula	Gomez et al. (2002)
Llama	Laparotomy	Artificial vagina, cooled to 5 °C	Percoll (90% and 45%)	Not reported	ICSI	SOFaa	Not reported	Morula	Miragaya et al. (2003)
Llama	Laparotomy/TUGA[b]	Anterior vaginal aspiration	Isotated (50% and 90%)	Not reported	ICSI	CR1aa Oviduct cells	7 7	8–16 cells Blastocysts	Sansinena et al. (2007)
Alpaca/Llama	Laparotomy	Llama epididymal[a]	Percoll (45% and 90%)	Not reported	IVF	Bovine oviduct cells	8	Morula	Ratto et al. (2007)
Alpaca	Slaughterhouse	Frozen epididymal[a]	Percoll (45% and 90%)	Not reported	IVF	SOFaa	7	Hatched blastocysts	Gamarra et al. (2008)
Llama	Laparotomy	Electroejaculation	After collagenase, Percoll (45%)	56.4 ± 17.5	ICSI IVF	SOFaa	7	Expanded blastocysts	Conde et al. (2008)
Alpaca	Slaughterhouse	Epididymal[a]	Percoll (22.5% and 45%)	Not reported	IVF	TCM199	2	2 cells	Huanca et al. (2009)
Alpaca	Laparotomy	Epididymal[a]	Percoll (45% and 22.5%)	Not reported	IVF	KSOM + SOF	7	Blastocysts	Huanca et al. (2010)
Alpaca	Slaughterhouse	Epididymal[a]	Percoll (22.5% and 45%)	Not reported	IVF	KSOM + SOF	7	Blastocysts	Condori et al. (2010)
Llama	TUGA[b]	Epididymal[a]	Percoll (45% and 90%)	Not reported	IVF		8	Blastocysts	Berland et al. (2011)
Llama	Laparotomy	Electroejaculation	After collagenase, Androcoll-E™	20–60	IVF	Cumulus cells SOFaa DMEM-F12	6	Hatched blastocysts Expanded blastocysts	Trasorras et al. (2010a)

[a] Slaughterhouse animals.
[b] Transvaginal ultrasound-guided oocyte aspiration.

(1%, 3/262) by IVF using slaughterhouse oocytes and frozen epididymal sperm. Our group has also worked with *in vitro* production of llama embryos using two assisted reproduction techniques: IVF (Conde et al., 2008; Trasorras et al., 2010a) and ICSI (Miragaya et al., 2003; Conde et al., 2008). The study reported by Conde et al. (2008) was the first to obtain expanded blastocyst after IVF or ICSI using gametes from live animals. The same study evaluated the effect of capacitating agents on IVF and oocyte activation after ICSI and a higher number of blastocysts were obtained by IVF without capacitating agents and by ICSI with oocyte activation using ionomicyn and DMAP. In our laboratory we obtained 9% hatched blastocysts by IVF without using capacitating agents (Trasorras et al., 2010a). Intracytoplasmic sperm injection was first carried out in llamas by Miragaya et al. (2003) using live animals. Oocytes were recovered surgically after superstimulatory treatment and live spermatozoa were separated by Percoll from ejaculates obtained using an artificial vagina. Injected oocytes were cultured in SOFaas media and 16% reached the morula stage. Sansinena et al. (2007) demonstrated that chemical activation of oocytes following ICSI improved the *in vitro* development of llama embryos, with ionomycin/DMAP activation resulting in higher proportions of 4–8 cell stages embryos cleaving after 36 h of culture. Nevertheless, the results obtained to date with regard to early embryo development are very low because the quality of the blastocysts produced *in vitro* are not comparable to that of blastocysts produced *in vivo*. This coincides with data published by Pope and Day (1972), Davis (1985) and Papaioannou and Ebert (1988) in pig, who observed that the number of blastomeres produced *in vitro* did not differ between different culture media but was in all cases lower than that observed for *in vivo* produced blastocysts. Although we have obtained pregnancies by ET using *in vivo* produced embryos (Trasorras et al., 2010b), the best quality *in vitro* produced llama embryos still have not produced pregnancies after transcervical transfer to the uterus of previously synchronized females. However, in the camel (*Camelus dromedaries*) this objective has been achieved after the transfer of *in vitro* produced embryos that were cultured in a semi-defined medium (Khatir and Anouassi, 2006). Applying this type of assisted reproductive techniques in genetically superior SAC would allow an increase in the number of superior embryos produced *in vitro* and would improve the reproductive efficiency of these species, both domestic and wild, in which the birth of a live *in vitro* produced offspring has not yet been achieved.

7. Nuclear transfer

The principle of nuclear transfer or cloning is relatively simple. The karyoplaste of a cell from the donor animal are transferred into the cytoplasm of an oocyte from which genetic material has been previously removed. The oocyte is enucleated through micromanipulation by removing the metaphase plate and polar body using a small-diameter pipette. The donor cell is then combined with the enucleated oocyte (cytoplast) by electro-fusion and the recombined oocyte is activated to stimulate embryonic development. This technique requires a large quantity

of oocytes. There is only one report of nuclear transfer in llamas and none in alpacas. Sansinena et al. (2003) used adult male llama fibroblast cell lines obtained from a skin biopsy and *in vitro* matured oocytes from superstimulated and ovariectomized females. Of a total of 80 reconstructed couplets, 62.5% were successfully fused, followed by cleavage rates of 32–40%. A total of 11 embryos (stage 8 cells to morula) were transferred to synchronized recipient llamas but no pregnancies were detected 14 days later. Nevertheless, the application of this technological advance was successful in the dromedary camel. Wani et al. (2010) obtained the first clone born alive of this species from a reconstructed embryo using cumulus cells.

8. *In vitro* culture of embryos

In vitro embryo culture can be done in co-culture of different types of cells or using defined or semi-defined synthetic culture media. The following have been used in different species: oviduct epithelial cells (Eyestone and First, 1989; Marquant-Le Guienne et al., 1989; Del Campo et al., 1994; Khatir et al., 2004), granulosa cells (Goto et al., 1989; Khatir et al., 2004), synthetic oviductal fluid (SOF) (Tervit et al., 1972; Takahashi and First, 1992; Carolan et al., 1999), Charle's Rosenkrans (CR) media 1 (Rosenkrans et al., 1993), CR media 2 (Tavares et al., 2002), potassium simplex optimization medium (KSOM) (Nedambale et al., 2002) and Dulbecco's modified Eagle's Medium (DMEM-F12) (Choi et al., 2004). According to Krisher et al. (1999) the use of co-culture with somatic cells is an acceptable technique for producing embryos. Nevertheless, this undefined medium makes it difficult to examine the nutritional requirements of the embryos and contributes to the variability in composition of the culture system (Bavister, 1995). In addition, the cells present in the culture medium can compete with the embryos for the nutrients or the metabolic waste can have a deleterious effect on embryo development. The use of defined culture media allows evaluation of the nutritional conditions necessary for the development of embryos to stages optimal for their transfer. The design of these media is based on embryo physiological and metabolical dynamics and on the reduction of intracellular stress. Furthermore, the data obtained from the oviduct microenvironment is taken into account. In SAC, no data has been published on the rate of production and the composition of the fluid produced by the oviduct and this is an area in need of research in this species. Early embryo development *in vivo* in llama seems to be rapid when compared to other species, such that morulas have been recovered *in vivo* from llama oviducts 3 days after ovulation (Miragaya et al., 2000). For this rapid growth to be possible, the contribution of an adequate microenvironment with nutrients, ions, hormones, proteins, amino acids and growth factors is necessary and probably present in the oviducts of these species.

Only one study reports the use of a defined culture medium (SOF) without co-culture with somatic cells to produce llama embryos *in vitro*, obtaining expanded blastocysts (17%, 16/94) (Conde et al., 2008) and we recently produced a 15% (5/33) expanded blastocysts after culture in DMEM-F12 (Trasorras et al., 2010a). In dromedary, two systems for *in vitro* culture have been compared with regard to their ability to support the development of dromedary oocytes to the blastocyst stage: semi-defined modified medium (mKSOMaa) and co-culture using camel epithelial oviduct cells. They showed a slight, but not significant, superiority of the semi-defined medium over the somatic cells co-culture system in terms of blastocyst formation, hatchability and pregnancy rate (Khatir et al., 2005). Berland et al. (2011) reported a 21% embryo development to the blastocyst stage, which was higher than that previously reported by Del Campo et al. (11%, 1994), but both studies used co-cultures with somatic cells. In Berland's work (2011), all embryos that developed to blastocyst failed to hatch and totally collapsed on day 8 of culture. In contrast, Del Campo et al. (1994) reported a 4.7% of zygotes reaching the hatched blastocyst stage and, in our laboratory we obtained a 9% of hatched blastocysts after 6 days of culture with SOFaa (Trasorras et al., 2010a).

All this research proves that it is possible to produce llama embryos *in vitro* but it remains necessary to find an adequate culture medium and conditions such as to favor embryo development to stages that are compatible with establishing pregnancy.

9. Conclusions

Since the beginnings of research in assisted reproductive techniques in SAC, where work was carried out on methods of synchronization and ovarian superstimulation, to latter years where studies have been conducted on *in vitro* embryo production, much progress has been achieved, being specially marked in the last nine years. Methods for oocyte maturation, IVF, ICSI and embryo culture to the blastocyst stage for ET have been developed and someday several of these techniques will be able to be applied in the field and others in reproduction centers. More researchers are needed to refine optimal systems for *in vitro* embryo production, especially to improve embryo development to stages that are compatible with establishing pregnancy, an objective which has not yet been attained in SAC.

Conflict of interest

None.

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