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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