Theriogenology 95 (2017) 127–132

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Effect of oocyte maturation time, sperm selection method and oxygen tension on *in vitro* embryo development in alpacas

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ARTICLE INFO

Article history:
Received 18 December 2016
Received in revised form
25 February 2017
Accepted 11 March 2017
Available online 16 March 2017

Keywords:
Alpaca
Oocytes
Embryos
Sperm selection
Oxygen tension

ABSTRACT

We evaluated the effect of *in vitro* maturation time, sperm selection and oxygen tension on alpaca embryo development. In Experiment I, Cumulus Oocyte- Complexes (COCs) were obtained from abattoir ovaries and *in vitro* matured in TCM-199 for 24 (n = 217), 28 (215), or 32 h (223) at 38.5 °C, high humidity and 5% CO_2 in air. Oocytes from 24 (n = 392), 28 (n = 456) or 32 (n = 368) h groups were *in vitro* fertilized with epididymal sperm and cultured in SOFaa at 38.5 °C, high humidity and 5% CO_2, 5% O_2 and 90% N_2 for 7 days. Embryo development was evaluated on Day 2, 5 and Day 7 of *in vitro* culture (Day 0 = *in vitro* fertilization). In Experiment II, a 2 by 2-factorial design was used to determine the effect of sperm selection (Swim-up vs Percoll) and oxygen tension (20% vs 5%) during embryo culture and their interaction on embryo development. COCs were *in vitro* matured for 32 h at 38.5 °C and 5% CO_2 in air and then *in vitro* inseminated with epididymal sperm processed by swim-up or Percoll. Zygotes were cultured in SOFaa + cumulus cells at 38.5 °C under 20 or 5% of O_2 tension and high humidity for 7 days. A total of 235, 235, 253 and 240 oocytes were assigned to: swim-up+20 O_2, swim-up+5 O_2 or Percoll+20 O_2, Percoll+5 O_2, groups respectively. The proportion of oocytes reaching MII stage was highest after 32 h of *in vitro* maturation ($P < 0.05$). Blastocyst rate (29.1 ± 2.7%) was also highest for COCs matured for 32 h (Exp I). In Experiment II, Blastocysts rate (26.03 ± 4.7; 27.7 ± 4.3; 29.7 ± 3.8 and 27.6 ± 4.2% for swim-up+20 O_2, swim-up+5 O_2 or Percoll+20 O_2, Percoll+5 O_2, respectively) was not affected by sperm selection method ($P = 0.8$), oxygen tension ($P = 0.9$) or their interaction ($P = 0.5$).

1. Introduction

The use of assisted reproductive technologies is in its formative stages in llamas and alpacas. Although, camelid embryos have been produced by *in vitro* fertilization (IVF) and nuclear transplantation, the birth of live offspring following the use of these techniques has not been reported to date. Indeed, many deficiencies of in *vitro* maturation, fertilization and embryo culture need yet to be overcome in order to standardize a consistent IVF protocol in these species (reviewed by Ref. [1]).

Only 5 IVF alpaca studies [1] have reported the development of morulas and blastocysts after *in vitro* fertilization of *in vitro* mature oocytes using epididymal sperm, and among laboratories, the *in vitro* maturation time required for the oocyte to reach the second metaphase (MII) has been quite variable. In a previous alpaca study [2], 84% of the oocytes reached the MII stage after *in vitro* maturation for 26 h. According to Huanca et al. [3], alpaca oocytes were able to reach nuclear maturation after 24 h of *in vitro* culture resulting in 19.3–22.2% of blastocyst development after *in vitro* fertilization. However, in those previous studies, oocytes were collected from superstimulated animals where the maturation process may be completely different from that of slaughterhouse's derived oocytes. One alpaca study has communicated that 65.8% of oocytes reached the maturation stage after 38 h of culture [4]. Indeed, the use of a prolonged *in vitro* maturation time (42 h) [5] involving an initial period of 21 h followed by a second phase of

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http://dx.doi.org/10.1016/j.theriogenology.2017.03.006
0093-691X/

22 h, with and without FSH supplementation respectively, has been reported to improve the maturation rate of alpaca oocytes. It has been difficult to compare and interpret the results among studies because of the diverse conditions used; therefore, a comparative study of *in vitro* maturation time will significantly contribute to the understanding of the first step of the complex process of IVF in this species.

On the other hand, llama or alpaca IVF has been performed using either epididymal sperm [2,6,7] or raw semen pretreated with collagenase to liquefy the high viscosity of the seminal plasma [8–10]. Moreover, motile llama sperm have been selected using either a Percoll gradient or more recently a silane-coated colloid (Androcoll-B [9]). Similarly, alpaca morula and blastocysts have been obtained with epididymal sperm selected by Percoll gradient [3–5,11]. Sperm selection method such as Swim-up or Percoll gradient provides morphological normal sperm populations with high progressive motility that may play an important role on alpaca *in vitro* embryo production. Although, bovine morula or blastocysts development were not affected when semen was processed by either Percoll or Swim-up method [12]; it was found that the rate of development, total cell number and number of inner cell mass were higher in those blastocyst produced by a commercial gradient colloid (BoviPure) or Percoll, than those produced by Swim-up [13–15]. It seems that the use of gradient centrifugation methods may select spermatozoa with more compacted chromatin and less nuclear DNA damage than Swim-up resulting in an increase on blastocyst development [16], clearly showing that sperm selection method impacts on the final outcome of embryo production.

Moreover, a potential endotoxic effect of Percoll [17] on bovine *in vitro* embryo production warrant further investigation [18]. None studies in alpacas have compared the effect of sperm selection methods on the outcome of in *vitro* embryo production.

Regarding to embryo development, llama and alpaca *in vitro* embryo culture has been developed adopting protocols used in ruminants such as somatic cells co-culture (cumulus or oviductal cells), or chemically defined media, with a range of 15–21% of blastocysts production [1]. In this regard, the effect of oxygen tension (20% vs 5%) during the *in vitro* embryo culture has not been evaluated in alpacas. In bovine, blastocyst development rate was greater after *in vitro* culture in low than higher oxygen tension [19,20]. Indeed, according to other reports the effect of co-culture with somatic cells on embryo development is more beneficial under low oxygen tension [21]. The benefits of low O_2 tension on embryo development and the deleterious effects of 20% O_2 in several species are well known [22]. Whether or not oxygen tension may affect the embryo development in alpacas requires further investigation.

Therefore, the main goals of the present study were to determine: 1) The effect of *in vitro* maturation time on nuclear maturation status and competence of alpaca oocytes collected from non-stimulated slaughterhouse derived ovaries (Experiment I), and 2) The effect of sperm selection method and oxygen tension on alpaca *in vitro* embryo development (Experiment II).

2. Materials and methods

All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, Mo, USA) unless stated otherwise.

2.1. Experiment I: the effect of in vitro maturation time on nuclear maturation status of alpaca oocytes obtained from non-stimulated ovaries

2.1.1. Ovaries, oocyte collection and in vitro maturation

Ovaries (n = 500) were obtained from adult non-pregnant alpacas during the breeding season (November–April) at a local slaughterhouse in Huancavelica, Peru, (12° 46′ S latitude, 74° 59′ W longitude; and 3680 m above sea level), and transported in phosphate buffered saline at 35 °C (PBS, Gibco) supplemented with penicillin (100 IU/ml, Gibco) and streptomycin (100 μg/mL, Gibco) to the laboratory. Cumulus-oocyte complexes (COCs) were aspirated from follicles 3–6 mm in diameter using a syringe attached to a 21-gauge needle containing PBS supplemented with penicillin (100 IU/mL), streptomycin (100 μg/L) and 0.3% bovine serum albumin (BSA). COCs were identified under a stereomicroscope at 15× magnification and categorized from Grade 1 to 5 according to the number of cumulus cell layers and the appearance of the oocyte cytoplasm [2,23–25]. Only grade 1 and 2 COCs were used in this study.

The COCs were *in vitro* matured as previously described [2,23]. Briefly, COCs were cultured in groups of 10–15 in 50 μl drops of TCM-199 (Gibco) supplemented with 10% heat-treated fetal calf serum (FCS, Gibco), 0.2 mM sodium pyruvate, 0.5 μg/mL FSH, 1 μg/mL estradiol-17β, and 25 μg/mL gentamycin. Drops were covered with mineral oil and cultured for 24, 28, or 32 h at 38.5 °C in a humidified atmosphere of 5% CO_2 in air.

2.1.2. Nuclear morphology assessment

Nuclear maturation status was evaluated in a sub population of COCs after 24, 28, 32 h of *in vitro* maturation (n = 217, 215, and 222 COCs, respectively). Nuclear morphology was assessed after denuding the COCs by repeated pipetting and fixation in 1:3 acetic acid/ethanol for at least 24 h. Denuded oocytes were stained with 1% orcein (w/v) in 45% acetic acid (v/v) and evaluated using phase-contrast microscopy at 400× magnification. Oocytes were classified as GV (presence of an intact germinal vesicle), GVBD (absence of nuclear membrane or nucleolus), MI (presence of a metaphase plate but no polar body), MII (presence of a metaphase plate and a polar body), abnormal or degenerated (i.e., oocytes with fragmented ooplasm, no distinct nuclear membrane or nucleolus, chromosomes dispersed in clumps within the ooplasm, or chromatin not recognizable at any stage of the meiosis [2,19]).

2.1.3. In vitro fertilization and embryo culture

COCs from 24 (n = 392), 28 (n = 456) or 32 (n = 368) h of *in vitro* maturation groups were fertilized *in vitro* using epididymal sperm obtained by the single sperm washing technique [26]. In brief, 6–7 alpaca epididymis were collected from mature males at the local slaughterhouse and transported on ice to the laboratory within 30 min of sacrifice. Epididymis tails were dissected and placed in Sperm-TALP (2 mM $CaCl_2$, 3.1 mM KCL, 0.4 mM $MgCl_2 \cdot 6H_20$, 0.3 mM $NaH_2PO_4 \cdot H_20$, 21.6 mM Lactic Acid (Sodium Salt; 60% w/w syrup), 100 mM NaCl, 1 mM Na Pyruvate, 25 mM $NaHCO_3$, 10 mM HEPES, 0.6% (w/v) BSA, Fraction V [27]). Sperm were recovered under stereomicroscopy by puncturing and squeezing the tissue, and aspirating with a 1 ml syringe attached to a 30-gauge needle. Sperm from 5 to 6 epididymis were then pooled and evaluated for motility. Samples (1.5 ml) with ≥75% progressive motility were centrifuged twice at 300 g for 6 min. Afterwards, the final pellet was suspended with Fert-TALP (2 mM $CaCl_2$, 3.2 mM KCL, 0.5 mM $MgCl_2 \cdot 6H_20$, 0.4 $NaH_2PO_4 \cdot H_20$, 11 mM Lactic Acid (Sodium Salt; 60% w/w syrup), 114 mM NaCl, 0.2 mM Na Pyruvate, 25 mM $NaHCO_3$, 20 mM Penicillamina, 10 mM Hypotaurine, 1 mM Epinephrine, 0.6% (w/v) fatty acid-free BSA [27]) and 10 μg/ml heparin to a final concentration of 1.0×10^6 spermatozoa/ml.

Expanded COCs (12–15) were washed with PBS supplemented with BSA, and then transferred into 50 μl drops of spermatozoa suspension and covered with paraffin oil. Gametes were co-incubated at 38.5 °C in air with 5% CO_2 and high humidity for 18 h. After *in vitro* fertilization, COCs were denuded by agitation for