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In vitro fertilization and development of cumulus oocytes complexes collected by ultrasound-guided follicle aspiration in superstimulated llamas

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Abstract

The objective was to evaluate the developmental competence of cumulus-oocyte complexes (COC) collected by follicular aspiration in llamas treated with FSH or eCG. Llamas were assigned randomly to two groups (n = 16 per group) and treated, at the time of ovarian follicular wave emergence, with either: 1) 25 mg of FSH im, twice daily for 4 d; or 2) 1000 IU of eCG as a single i.m. dose. The start of gonadotropin treatment was considered Day 0. Both groups were given 5 mg of Armour Standard LH im on Day 6, and COC were collected by follicle aspiration on Day 7. Expanded COC collected from FSH- (n = 157) and eCG-treated llamas (n = 151) were fertilized *in vitro* using epididymal sperm, and presumptive zygotes were *in vitro* cultured in SOF medium for 8 d. The FSH and eCG treatment groups did not differ with respect to: the number of follicles ≥7 mm (16.0 ± 2.7 vs 14.0 ± 1.9, respectively; P = 0.5); the number of COC collected (11.5 ± 1.9 vs 9.7 ± 1.2; P = 0.4); the number of expanded COC (9.8 ± 1.4 vs 9.4 ± 1.2; P = 0.8); or the percentage of presumptive zygotes which developed into 2 to 8 cell stage embryos (65.3 vs 63.1), morulas (46.2 vs 42.5), or blastocysts (23.1 vs 20.5; P > 0.05). In conclusion, FSH and eCG treatments were equally effective for recovery of a high number of expanded COC which were used directly for *in vitro* fertilization. Furthermore, rate of embryo development was not significantly affected by the gonadotropin treatment used.

Keywords: Oocyte competence; Superstimulation; in vitro fertilization; Camelids; Gonadotropins

1. Introduction

Since the report of the first llama cria by nonsurgical procedures [1], embryo transfer technology has been applied with limited success in South American camelids. *In vivo* production of llama/alpaca embryos can be achieved by nonsurgical uterine flushing after gonadotrophin superstimulatory treatment [2], or by the recovery of a single embryo in non-superstimulated females [3,4]. Several limiting steps have been associated with the slow development of the embryo transfer

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technique in camelids, including extremely variable follicular responses after superstimulatory treatment [2], and inconsistent results regarding semen preservation and AI [5]. Furthermore, recovery of embryos after uterine flushing in hatched blastocyst stage made it difficult to apply other technologies, e.g., cryopreservation techniques, thereby limiting the potential for international trade of camelid [6,7]. Development of an *in vitro* embryo production system in camelids may overcome some of the problems associated with embryo transfer. However, improvements in oocyte maturation, fertilization, and embryo culture are necessary to achieve success in development of any *in vitro* fertilization program.

The first llama embryos were produced *in vitro* using Cumulus Oocyte Complexes (COC) collected by follicular puncture from abbatoir-derived ovaries [8]; this was followed by the first *in vitro* production of alpaca and llama embryos from COC collected by surgical follicular aspiration from females superstimulated with gonadotrophin [9,10]. Regarding other camelid species, e.g. dromedary, blastocysts have been produced *in vitro* using COC collected by follicle puncture form abattoir-derived ovaries [11,12]. Although the first dromedary offspring was obtained by transfer of *in vitro* hatched blastocysts to synchronized recipients [13], there are not apparently reports documenting the births of llamas or alpacas produced with this technique. However, development and application of transvaginal ultrasound-guided follicular aspiration in llamas and dromedary during the last 10 y [14–17] to collect COC from i*n vivo* superstimulated and non-superstimulated females will facilitate the use of this technique as a valuable tool to maximize the genetic potential of these species.

Ovarian superstimulation for oocyte collection in alpacas [18], llamas [10,16,19,20] and vicuñas [21], with either FSH or a single dose of eCG, followed by GnRH or LH, have resulted in a high recovery rates of expanded COC. As a result of the long biological half-life of eCG, ovarian superstimulatory protocols based on a single dose of eCG have the advantage of minimizing animal handling and stress compared to multiple-dose protocols based on FSH. The long half-life of eCG, however, has been associated with premature maturation of bovine oocytes during superstimulatory treatment, resulting in deleterious effects on oocyte quality and subsequent embryo development [22–24]. In a more recent bovine study [25], eCG treatment resulted in the recovery of a fewer good quality COC than FSH treatment.

In a previous study [16] no differences were detected between FSH- and eCG-treated llamas in the number of expanded COC collected (8.3 ± 2.1 vs 10.6 ± 2.2) or the number of COC at the MII stage (6.9 ± 1.8 vs 8.9 ± 1.9). In a similar study in alpacas [18], FSH- and eCG-treated animals did not differ with respect to the number of follicles > 6 mm at the time of COC collection (20.0 ± 7.5 vs 27.0 ± 3.3), the number of COC collected (26.2 ± 8.4 vs 23.3 ± 3.7), number of expanded COC collected (11.5 ± 2.9 vs 8.8 ± 2.8), or the number of expanded COC in MII (8.5 ± 1.9 vs 6.0 ± 2.1). However, a direct comparison between FSH and eCG treatment on oocyte competence in llamas after *in vitro* fertilization has apparently not been reported.

The objective of the present study was to compare developmental competence of COC collected by ultrasound-guided follicular aspiration in llamas treated with FSH or eCG.

2. Materials and methods

2.1. Animals and treatments

Mature, non-pregnant female llamas (n = 32), ≥ 3 y of age and weighing an average of 120 kg, were used during the breeding season (January to March) at the Llama del Sur Ranch, in the Department of Temuco, Chile (38° S, 72° W, at sea level). To synchronize follicular wave emergence, females were given caudal epidural anesthesia (2.5 mL of 2 % of lidocaine; Laboratorio Chile, Santiago, Chile), restrained in dorsal recumbency, and all ovarian follicles ≥ 5 mm were ablated by transvaginal ultrasound-guided follicle aspiration, using a 5.0 MHz convex-array ultrasound transducer (Aloka SSD-500, Tokyo, Japan) and a 19-gauge needle [14–16]. At 48 h after follicle ablation [i.e., expected time of follicular wave emergence; (15)], llamas were assigned randomly to two groups (n = 16 per group) and given either: 1) 25 mg of FSH (Folltropin, Bioniche Animal Health Canada Inc., Belleville, ON, Canada) i.m., twice daily for 4 d; or 2) 1000 IU of eCG (Novormon, Bioniche Animal Health Canada Inc.) as a single im dose. The start of gonadotropin treatment was considered Day 0. Llamas in both groups were given 5 mg Armour Standard LH (Lutropin, Bioniche Animal Health Canada Inc.) i.m. on Day 6. The ovarian response was assessed by transrectal ultrasonography, using a 7.5 MHz linear-array transducer (Aloka SSD-500) immediately before COC collection on Day 7 (24 to 26 h after LH treatment).

Table 1
Ovarian follicular response and transvaginal ultrasound-guided collection of cumulus-oocyte complexes (COC) in llamas superstimulated with FSH or eCG given at follicular wave emergence, followed by LH treatment (mean ± SEM and proportions).

Treatment group	Follicles detected (≥ 7 mm)	Follicles aspirated*	COC collected	COC recovery rate†
FSH (n = 16)	16 ± 2.7	240/257 (93.3%)	11.5 ± 1.9	185/240 (77.0%)
eCG (n = 16)	14 ± 1.9	218/225 (96.8%)	9.7 ± 1.2	156/218 (71.5%)

No differences between groups for any endpoint (P = 0.4).
* Number of follicles (≥ 7 mm) aspirated over the total number of follicles (≥ 7 mm) detected.
† Number of COC collected over the number of follicles aspirated.

2.2. Collection and classification of COC

Cumulus-oocyte complexes were collected by transvaginal ultrasound-guided aspiration of follicles ≥ 7 mm on Day 7, as previously described [15,16]. Follicular contents were aspirated using a regulated vacuum pump set at a flow-rate of 22 mL/min. Follicular contents were aspirated into a 50 mL conical tube containing phosphate buffered saline (PBS) with 0.3% bovine serum albumin (BSA), heparin (10,000 IU/L PBS) and gentamycin (50 μg/L PBS). Aspirates were transferred to petri dishes and a stereomicroscope was used to locate and evaluate COC. The COC were classified as expanded, compact (≤4 layers of granulosa cells tightly surrounding the oocyte), denuded, or degenerated (pyknotic granulosa cells and vacuolated ooplasm) [16].

2.3. In vitro fertilization and embryo culture

Expanded COC collected from llamas treated with FSH or eCG were fertilized *in vitro* using epididymal llama sperm, as previously described [8,9]. In brief, llama epididymides were collected from mature males at an abattoir and transported on ice in a thermos to the laboratory within 25 min after slaughter. The tissue temperature upon arrival was 5 to 7 °C. Cauda epididymides were dissected and placed in a small petri dish containing Sperm-TALP media supplemented with 3 mg/mL of BSA [26]. Sperm was recovered under stereomicroscopy by puncturing and squeezing the tissue using a 30-gauge needle attached to a 1 mL syringe. Sperm from five or six cauda epididymides were pooled and evaluated for motility. Samples with ≥ 75% progressive motility were centrifuged in a discontinuous percoll gradient (1 mL of 45% over 1 mL of 90% percoll) for 15 min at 700 g. The supernatant was removed and the pellet was suspended with Sperm-TALP and centrifuged at 350 g for 6 min. The supernatant was removed and the final pellet was suspended with Fert-TALP [26] supplemented with 6 mg/ml of fatty acid-free BSA (Sigma) and 10 μg/mL heparin (Sigma), to a final concentration of 1 x 10^6 sperm/mL. Expanded COC were washed with PBS supplemented with BSA, and then transferred into a small petri dish with a 50 μL drop of sperm suspension and covered with mineral 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, COC were vortexed for 3 min to remove the cumulus cells, washed three times in PBS with BSA, and finally cultured in SOF medium supplemented with 0.6% of BSA and cumulus cells at 39 °C, 5% CO_2, 5% O_2, 90% N_2 for 8 d. Presumptive zygotes recovered from llamas treated with FSH and eCG were transferred to culture medium. Embryo development was evaluated by stereomicroscopy at 2, 5, 7, and 8 d after IVF.

2.4. Statistical analyses

Means were compared between the FSH and eCG treatment groups with Student's *t*-tests, and proportional data were compared between groups with Fisher's exact test. For all analyses, $P < 0.05$ was considered significant.

3. Results

There were no significant differences between FSH- and eCG-treated groups in the number of follicles ≥ 7 mm at COC collection, the number of follicles aspirated, the number of COC collected per llama, or the proportion of COC recovered (Table 1). Furthermore, there was no difference between groups in the number of expanded COC collected per llama (P = 0.8), but more compact COC were collected from llamas treated with FSH ($P < 0.01$; Table 2). A total of 157 and 151 expanded COC collected from llamas treated with FSH or eCG respectively were fertilized *in vitro* using epididymal sperm; however, only 147 and 141 of the zygotes from FSH or eCG-treated groups, respectively, were submitted to embryo culture (the remainder were lost during the previous vortexing and washing). The

Table 2
Morphologic characteristics (mean ± SEM) of cumulus-oocyte complexes (COC) collected from llamas superstimulated with FSH or eCG at follicular wave emergence followed by LH treatment.

Treatment group	COC collected	Expanded COC	Compact COC ≥4 layers
FSH (n=16)	11.5 ± 1.9	9.8 ± 1.4	2.1 ± 0.5[a]
eCG (n=16)	9.7 ± 1.2	9.4 ± 1.2	0.5 ± 0.2[b]

[a,b] Within a column, means without a common superscript differed ($P < 0.01$).

proportion of presumptive zygotes that developed to the 2 to 8 cell stage (2 d after IVF), morula stage (5 d), and blastocyst stage (7 d) did not differ between FSH and eCG-treated groups (Table 3, Fig. 1). All blastocysts in both treatment groups had well developed blastoceles by 7 d after IVF (Fig. 2), but none had hatched by 7 or 8 d of culture. Most of the blastocysts in both groups collapsed on the 8th day of *in vitro* culture.

4. Discussion

In the present study, superstimulatory treatments (FSH or eCG) were equally effective for ovarian superstimulation and oocyte collection. The recovery of a high number of expanded COC could be used directly for *in vitro* fertilization (their competence was not affected by gonadotropin treatment).

Since the superstimulatory response was relatively consistent among animals in both groups, we inferred that the variability to the response could be partly controlled when gonadotrophin treatments were initiated at follicular wave emergence [15,16,27,28]. Based on previous work in llamas [15], follicular wave emergence was expected 2.3 ± 0.3 d after follicular ablation; hence, treatment was initiated 2 d after ablation in the present study.

In ruminants, there are huge variations among individual animals in their ovarian response to superstimulatory protocols; some of this variation was attributed to the type of gonadotropin preparation used [22]. In that regard, the long half-life of eCG makes it persist to persist for up to 10 d in the bovine circulation, inducing continuous follicular growth and abnormal endocrine profiles and resulting in unovulatory or luteinized follicles that can affect oocyte competence [29,30]. Moreover, ovarian superstimulation with eCG for COC collection in cattle resulted in the recovery of more bad quality oocytes than in those given FSH [25].

In the present study, numerous expanded COC were recovered from llamas superstimulated with either eCG or FSH. We previously documented [16] that ~80% of the expanded COC recovered from llamas treated with gonadotrophin and LH were in second metaphase. The total mean number of expanded COC form eCG-treated llamas seemed similar to that reported in our previous studies conducted in llamas and alpacas [16,18], whereas there were apparently more expanded COC from FSH-treated llamas in the present study compared to previous studies [16,18]. We speculated that this was related to the use of a longer interval from LH administration to follicular aspiration in this study (24–26 h), compared to the 20–22 h interval used in the former studies. The recovery of expanded COC could facilitate and improve the *in vitro* fertilization system, since there was no need for *in vitro* maturation.

In recent studies in llamas, there were many expanded COC collected when females were treated with 1000–1500 IU of eCG [10,20]. Moreover, the use of a single dose of 1000 IU of eCG plus 8 ug of Buserelin [10] resulted in recovery of 193 expanded COC from 223 aspirated follicles (86.5% recovery rate). Overall, 146 expanded COC were submitted for embryo production, resulting in 17% (16/94) and 9.6% (5/52) of blastocysts development after *in vitro* fertilization and ICSI respectively, indicating that eCG did not compromise oocyte competence in term of blastocyst formation. The percentage of blastocysts in the present study (~21%) seemed higher than that reported previously (11%; [8]) using COC collected by follicular puncture from abbatoir-derived ovaries. In contrast to the present study, 4.7% of zygotes reached hatched blastocyst stages after co-culture with llama oviductal epithelial cells [8]. In a dromedary study [12], there were 21 and

Table 3
Development of expanded cumulus-oocyte complexes collected from llamas superstimulated with FSH or eCG, after *in vitro* fertilization with epididymal sperm, followed by *in vitro* culture in SOF medium supplemented with 0.6% bovine serum albumin and cumulus cells.

Treatment group*	Zygotes	2–8 cells	Morulas	Blastocysts
FSH (n = 16)	147	96/147 (65.3%)	68/147 (46.2%)	34/147 (23.1%)
eCG (n = 16)	141	89/141 (63.1%)	60/141 (42.5%)	29/141 (20.5%)

* No differences between groups for any endpoint ($P = 0.8$).

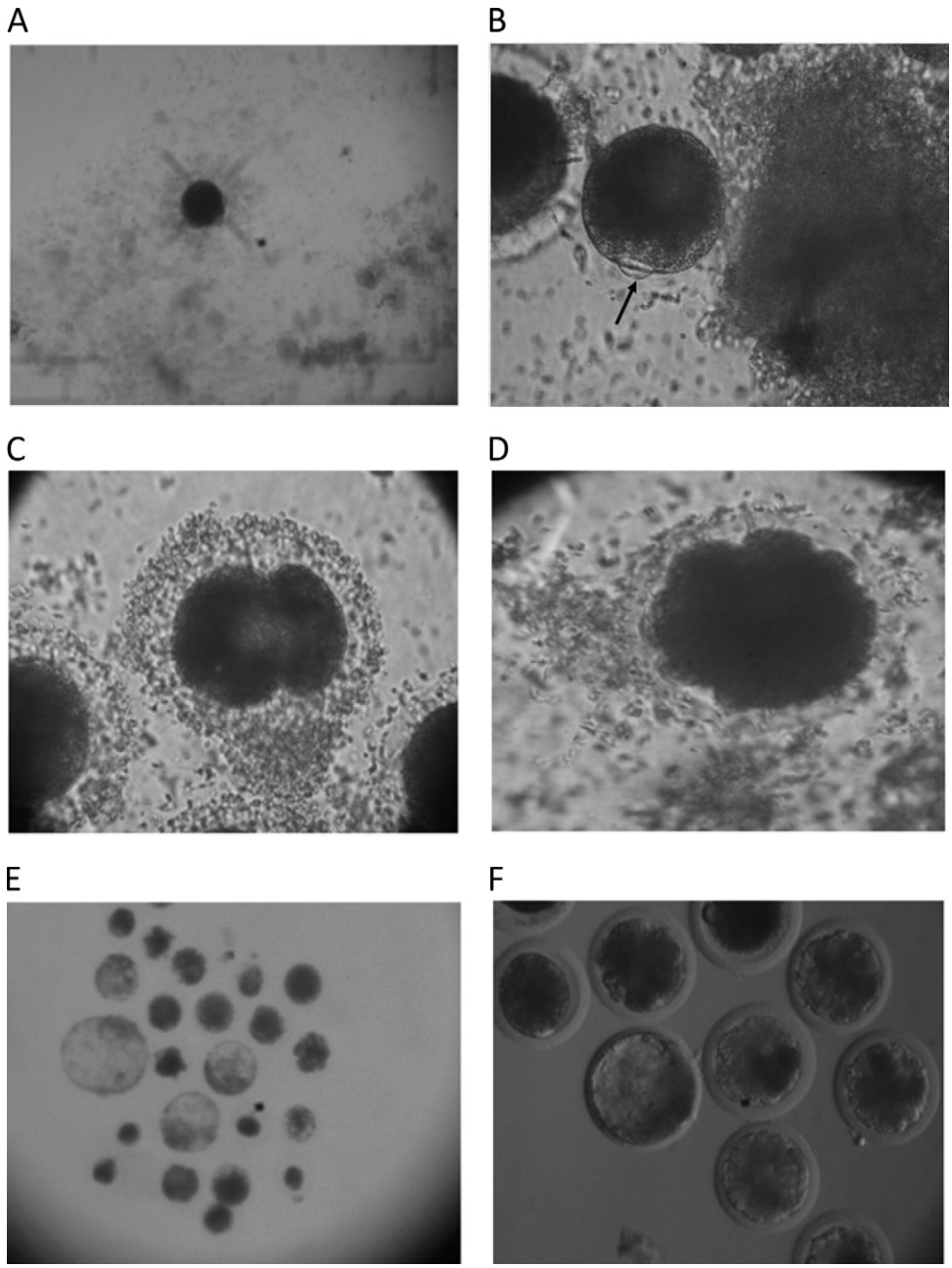

Fig. 1. Morphologic sequence of cumulus-oocyte complexes (COC) collected from llamas superstimulated with FSH or eCG, followed by LH. (A) Expanded COC recovered by ultrasound-guided follicular aspiration after LH treatment. (B) Mature llama oocyte (arrow indicates the polar body). (C) two-cell embryo 2 d after *in vitro* fertilization. (D) Compact morulas 5 d after *in vitro* fertilization. (E & F) Compact morulas and expanded blastocysts 7 d after *in vitro* fertilization.

16.5% blastocysts, and 21 and 14% hatched blastocysts produced when zygotes were cultured in either semi-defined medium mKSOMaa or oviductal epithelial cells respectively. In a recent study in dromedary [17], there was a higher proportion of blastocyst development (50%) after *in vitro* fertilization of COC collected by ultrasound-guided follicular aspiration from superstimulated females (given a combination of 2500 IU of eCG plus 400 mg of FSH, followed by GnRH) than that from COC submitted for *in vitro* maturation (30%).

In the previous study [10], semen was collected by electroejaculation and treated with collagenase to facil-

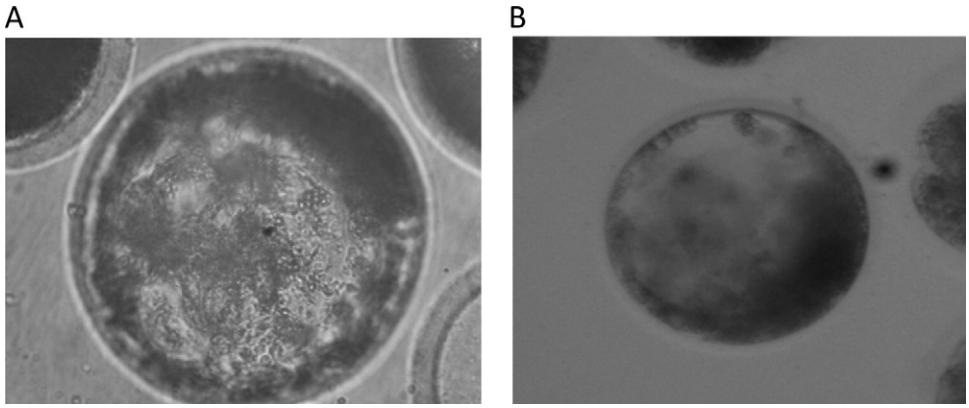

Fig. 2. Blastocyst development 7 d after *in vitro* fertilization of expanded COC collected from llamas treated with gonadotrophin, followed by LH administration. (A) Blastocysts developed in SOF medium from FSH-treated llamas. (B) Blastocysts developed in SOF medium from eCG-treated llamas.

itate sperm manipulation during *in vitro* fertilization. Although this protocol enabled work with mature sperm and could improve the rate of *in vitro* fertilization and embryo development in llamas, the percentages of embryo cleavage on Day 2 and blastocyst formation on Day 7 in the present study seemed higher than those previously reported [10]. Although it has been reported that collagenase did not affect both motility and sperm membrane functionality of the llama sperm [31], it will be necessary to conduct further investigations to determine whether this enzyme can affect the sperm membrane, or alter sperm function, and whether this could affect the *in vitro* fertilization process and subsequent embryo development.

In contrast to results reported by other investigators working with camelids [8,12,13], all blastocysts developed on Day 7 in this study failed to hatch; they totally collapsed on Day 8. Therefore, in future studies, it will be necessary to clarify whether the use of epididymal sperm or the *in vitro* culture conditions affected *in vitro* fertilization or embryo development.

In conclusion, numerous expanded COC were collected and used directly for *in vitro* fertilization. Furthermore, their competence was not affected by gonadotrophin treatment.

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