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Oocyte production and *in vitro* maturation in Canindé goats following hormonal ovarian stimulation

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Abstract

This study evaluated the effect of hormonal ovarian stimulation regimes on the quantity and quality of the *cumulus*-oocyte complexes (COCs) recovered by laparoscopy and their subsequent *in vitro* maturation (IVM). Eighteen cyclic Canindé goats received a vaginal sponge with 60 mg medroxyprogesterone acetate for 11 days, together with an injection of 50 µg d-cloprostenol on the 8th day, along with additional treatment regimens, as follows: i) five doses (5D), 120 mg of NIH-FSH-P1 in five injections at 12 h intervals; ii) three doses (3D), 120 mg of NIH-FSH-P1 in three injections at 24 h intervals; iii) single dose (1D), 70 mg of NIH-FSH-P1 and 200 IU of eCG at 36 h prior to sponge removal. Three sessions of hormonal treatment/oocyte recovery were performed and goats (n = 6/group) were allocated to different groups. The oocytes were collected by laparoscopy at the time of sponge removal and the IVM of the oocytes was monitored. A total of 14.8 ± 0.8 follicles were aspirated per animal with 11.1 ± 0.6 COCs being recovered, resulting in a recovery rate of 74.7% (577/772). The mean number of COCs/goat did not differ ($P > 0.05$) between treatments (11.7 ± 1.1, 10.7 ± 0.9 and 10.8 ± 1.0 for the 5D, 3D and 1D groups, respectively). Recovery rate was higher ($P < 0.05$) in the 5D group (84.1%; 211/251) compared to the 3D (68.2%; 182/267) and 1D groups (72.4%; 184/254). The lowest ($P < 0.05$) maturation rate was recorded in the 3D group (32.1%; 27/84), while the rate for the 5D and 1D groups was 49.1% (53/108) and 46.2% (42/91), respectively. Finally, taking into account the main performance results of the three treatments, it is advisable to use the 5D regime in future Canindé breed preservation programs based on laparoscopy technology as a means to recover oocytes.

Keywords: endangered species, gonadotropin, *in vitro* maturation, laparoscopy, oocyte.

Introduction

The goat population in Northeastern Brazil is approximately 8.7 million, corresponding to 91% of the total number of goats in Brazil (Brazilian Institute of Geography and Statistics, 2007). The northeastern population is mostly made up of undefined breed types. There are also some herds of animals derived from imported high producing milk breeds (e.g. Saanen, Alpine, etc.), as well as herds with naturalized highly adapted breed types. Among these, naturalized breeds (Canindé and Moxotó) play an important role in subsistence stock farming, although the uncontrolled crossbreeding of these breeds with exotic breeds has caused genetic degeneration, placing the indigenous breeds at risk of extinction (Mariante and Egito, 2002).

Modern reproductive biological techniques, such as artificial insemination, embryo transfer and *in vitro* embryo production (IVEP) have exhibited great potential to preserve endangered breeds (Long, 2008). The IVEP method involves three basic steps, namely *in vitro* maturation (IVM) of primary oocytes collected from antral follicles, fertilization of matured secondary oocytes and culture of potential probable embryos to the blastocyst stage. These embryos can then be transferred to recipient females or cryopreserved for future use (Paramio, 2010). In this whole process, two factors are of key importance: obtaining high quality oocytes and successful IVM of the oocytes (Zhou *et al*., 2008).

To obtain high quality oocytes, laparoscopic oocyte recovery (LOR) has been extensively used in goats (Gibbons *et al*., 2007; Leoni *et al.*, 2009). LOR is minimally invasive and allows for a rapid recovery of females after oocyte collection. Additionally, laparoscopy poses low risks for the development of adhesions, enabling repeated collections from the same animal in a short period of time (Pierson *et al*., 2004).

Hormonal treatment for ovarian stimulation is often used prior to oocyte recovery in order to increase the number of aspirated follicles (Baldassarre *et al*., 2003; Pierson *et al*., 2004). Indeed, the number of follicles available and the subsequent number of *cumulus*-oocyte complexes (COCs) recovered would be substantially reduced without hormonal treatment (Aguilar *et al*., 2002).

However, in goats the best ovarian stimulation treatment prior to oocyte recovery is still to be

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defined. Furthermore, for Brazilian naturalized goat breeds there is little or no information in the literature regarding the use of hormonal treatments for ovarian stimulation. Therefore, the aim of this study was to compare three different treatments by observing their effect on the number of follicles induced for aspiration, the number and quality of COCs recovered, and the subsequent maturation rate of oocytes submitted to IVM for the naturalized and endangered Canindé goat breed.

Materials and Methods

Location and experimental animals

The experiment was conducted in the Laboratory of Physiology and Control of Reproduction (LFCR; Faculty of Veterinary, State University of Ceará) located in Fortaleza, CE, Brazil, at 3º47'38"S and 38º33'29"W. A total of 18 cyclic Canindé goats (mean live weight ± SEM, 32.9 ± 0.5 kg) were selected as oocyte donors. All animals were housed and maintained in a semi-intensive system, receiving Tifton (*Cynodon dactylon*) hay in pens and having 6 h of daily access to pasture. In addition, animals were supplemented with commercial concentrate (minimum of 20% crude protein) and free access to water and minerals. All sampling frequencies and handling procedures were designed to minimize stress for subjects, according to the current guidelines for the ethical use of animals in research (Association for the Study of Animal Behaviour, 2006).

Chemicals, reagents and media

All chemicals and media used were supplied by Sigma Chemical Company (St. Louis, MO, USA), unless otherwise stated. When necessary, for all media, the pH and osmolarity were adjusted to between 7.2 to 7.4 and 280 to 300 mOsm/kg, respectively.

Hormonal treatments

All oocyte donors received intravaginal sponges containing 60 mg medroxyprogesterone acetate (MAP; Progespon, Syntex, Buenos Aires, Argentina) for 11 days, combined with an intramuscular (i.m.) injection of 50 µg d-cloprostenol (Ciosin, Coopers, São Paulo, Brazil) on day 8 of progestagen treatment. For ovarian stimulation, goats were allotted into one of the following experimental groups: i) five doses (5D) of 120 mg NIH-FSH-P1 (Folltropin-V, Vetrepharm, Belleville, Canada) applied as five i.m. injections (30/30, 20/20 and 20 mg) at 12 h intervals; ii) three doses (3D) of 120 mg NIH-FSH-P1, administered as three i.m. injections (60, 40 and 20 mg) at 24 h intervals; iii) single dose (1D) as a single i.m. injection of 70 mg NIH-FSH-P1 plus 200 IU eCG (Novormon, Syntex, Buenos Aires, Argentina) 36 h before sponge removal. The pFSH injections in the 5D and 3D groups started on day 8 of progestagen treatment. The pFSH doses were established based on previous studies carried out on the superovulation of these naturalized goat breeds by our group (Souza *et al*., 2008; Moura *et al*., 2010). The dose from the 1D treatment was based on previous studies with goats performed by Baldassarre *et al*. (2003). Thus, there were three successive hormonal treatments/LOR session at intervals of 14 days. For each session, the goats were allocated to a different experimental group to avoid the effect of treatment repetition.

Anesthesia and LOR

The females were deprived of feed and water for 36 h and 24 h, respectively, prior to laparoscopy. Animal suffering was avoided by implementing laparoscopy under anesthesia with 0.5 mg/10 kg of xylazine hydrochloride (Coopazine, Coopers, São Paulo, Brazil) and 25 mg/10 kg of ketamine hydrochloride (Ketamina Agener, União Química, Embu-Guaçu, Brazil) associated with local administration of 2% Clorhydrate lidocain (Anestésico L Pearson, Eurofarma, Rio de Janeiro, Brazil) applied to the puncture sites of the trocars. The LOR procedure was performed just after sponge removal by using a 5-mm laparoscope (Karl Storz Endoscopes GmbH & Co., Tuttlingen, Germany) attached to a video system. Briefly, the laparoscope was inserted into the abdominal cavity via a trocar cranial to the udder and to the left of the midline. A non traumatic grasper was also inserted into the right side of the abdomen using a trocar. The ovary was held by the grasper and ovarian follicles were individually aspirated using a 22-gauge needle connected to an aspiration and flushing system (WTA, Cravinhos, Brazil). The vacuum pressure was set at 30 mmHg, generating a fluid flow of 7 to 7.5 ml/min. All follicles larger than 2 mM were aspirated. The collection medium used was TCM199 supplemented with HEPES (10 mM), heparin (20 IU/ml) and gentamicin (40 µg/ml). Once the LOR was completed, each ovary was gently flushed with a heparinized saline solution (25 IU/ml) at 37ºC for the prevention of possible adhesions. Finally, the trocar orifices were treated with a local antibiotic healing solution (Terra-cortril, Pfizer, Porto Alegre, Brazil).

Assessment of COC quality and IVM

Assessment of the quality of the COCs was based on visual criteria (SMZ 800, Nikon, Tokyo, Japan), allocating different grades (1 to 4) based on our experience with Brazilian naturalized breeds (Table 1) using a stereomicroscope. Grades 1 and 2 were classified as good quality COCs for IVM. The remaining oocytes classified as grades 3 and 4 were discarded. These selected COCs were then washed four

times in IVM medium consisting of TCM199 supplemented with cysteamine (100 µm), epidermal growth factor (EGF, 10 ng/ml) and gentamicin sulfate (40 µg/ml). Good quality COCs were incubated (38.5°C) in 500 µl of IVM medium in a humidified atmosphere with 5% CO_2 in air for 24 h. After the IVM period, the COCs were visualized under an inverted microscope (TE2000, Nikon, Tokyo, Japan) and the *cumulus* cells were carefully removed by successive pipetting. Denuded oocytes were assessed for maturation. Oocytes with a visible first polar body were considered to be mature and meiotically competent.

Table 1. Criteria for grading *cumulus*-oocyte complexes (COCs) recovered from Canindé goats.

Characteristics	Grade
Multilayered compact cumulus and finely granulated oocyte cytoplasm	1
One to three layers of cumulus cells and finely granulated oocyte cytoplasm	2
Incomplete or no cellular investment or heterogeneous oocyte cytoplasm	3
Oocyte with abnormal shape and heterogeneous oocyte cytoplasm or apoptotic oocytes in jelly-like cumulus-corona cells investment	4

Statistical analyses

Follicle aspiration and oocyte recovery data were analyzed by ANOVA Mixed Models Procedure, SAS/STAT, 1997. Proportional data were compared using Fisher's exact test. All results were expressed as the mean ± SEM and statistical significance was accepted at a confidence level of $P < 0.05$.

Results

Just after the first LOR, one of the goats in the 5D group died. Therefore, in the subsequent sessions the number of experimental animals declined to five in the 3D group (second session) and in the 1D group (third session). At the end of the three hormonal treatments/LOR sessions, a total of 881 follicles had been visualized and 772 of them aspirated. A mean of 14.8 ± 0.8 aspirated follicles and 11.1 ± 0.6 COCs were recorded from each donor goat, resulting in an overall oocyte recovery rate of 74.7% (577/772).

In Table 2, a summary of the results following ovarian stimulation is shown. No differences were recorded between the hormonal treatments tested regarding the mean number of follicles visualized and aspirated on each ovary, and overall. The mean number of COCs recovered per donor did not differ between treatments and was 11.7 ± 1.1, 10.7 ± 0.9 and 10.8 ± 1.0 for the 5D, 3D and 1D groups, respectively. However, the recovery rate of the 5D group (84.1%; 211/251) was higher ($P < 0.05$) than for the 3D group (68.2%; 182/267) and the 1D group (72.4%; 184/254).

Table 2. Effect of different hormonal ovarian stimulation regimes on the number of visualized and aspirated follicles (mean ± SEM) and *cumulus*-oocyte complex (COC) recovery rate in Canindé goats.

Hormonal treatment	n	Visualized follicles			Aspirated follicles			Recovery rate (%)
		Right ovary	Left ovary	Total	Right ovary	Left ovary	Total	
5D	18	8.5 ± 0.8[a]	8.3 ± 0.9[a]	16.8 ± 1.5[a]	6.7 ± 0.6[a]	7.2 ± 0.8[a]	13.9 ± 1.3[a]	84.1[a]
3D	17	8.5 ± 0.8[a]	8.5 ± 0.8[a]	17.1 ± 1.4[a]	8.1 ± 0.8[a]	7.6 ± 0.8[a]	15.7 ± 1.3[a]	68.2[b]
1D	17	7.4 ± 0.8[a]	9.6 ± 0.9[a]	17.0 ± 1.4[a]	6.6 ± 0.8[a]	8.3 ± 0.9[a]	14.9 ± 1.4[a]	72.4[b]

Values in the same column with different superscripts differ significantly ($P < 0.05$).

Following oocyte recovery and morphological evaluation of the COCs (Fig. 1), all oocytes were classified into different grades (1 to 4). No difference was observed between the ovarian stimulation treatments, except for the grade 4 COCs which were more frequent ($P < 0.05$) in the 3D group (Fig. 2). Regarding the viable COCs (grades 1 and 2), there was no difference between the treatments. The percentages of oocytes classified as viable and thus used in the IVM procedure were 73.9% (156/211), 66.4% (121/182) and 70.1% (129/184) for the 5D, 3D and 1D groups, respectively.

Approximately half of the COCs in each group were removed for use in another experiment in the laboratory. This was done taking into account the different proportions of the different quality grades in the three groups. Therefore, the number of grade 1 and 2 COCs subjected to IVM from each group was 108 (5D), 84 (3D) and 91 (1D). After IVM, there were differences ($P < 0.05$) regarding the maturation rates achieved among treatments. The lowest ($P < 0.05$) maturation rate was verified in oocytes from the 3D group (32.1%; 27/84), compared to the maturation rates from the 5D (49.1%; 53/108) and 1D (46.2%; 42/91) groups.

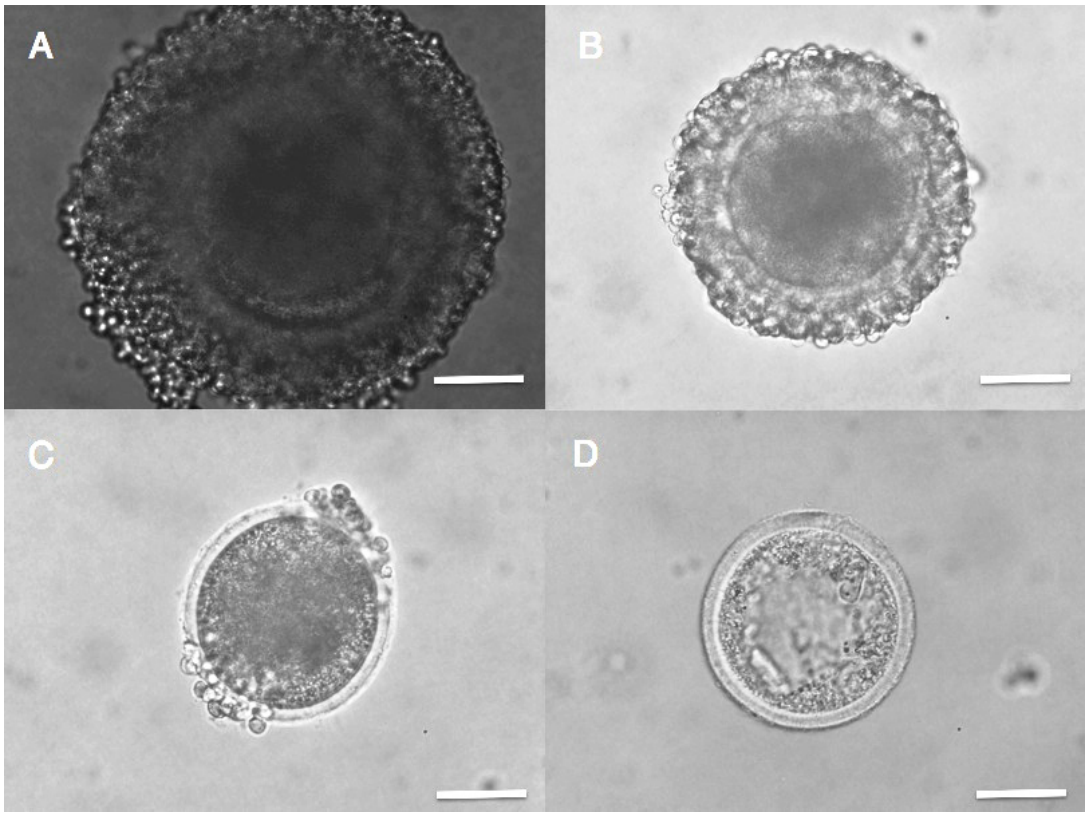

Figure 1. Typical *cumulus*-oocyte complexes (COCs) recovered from Canindé goats receiving different hormonal ovarian stimulation regimes. COCs are classified as grade 1 (A), grade 2 (B), grade 3 (C) and grade 4 (D). Grades 1 and 2 were selected for IVM and grades 3 and 4 were discarded. Scale bar represents 50 µm.

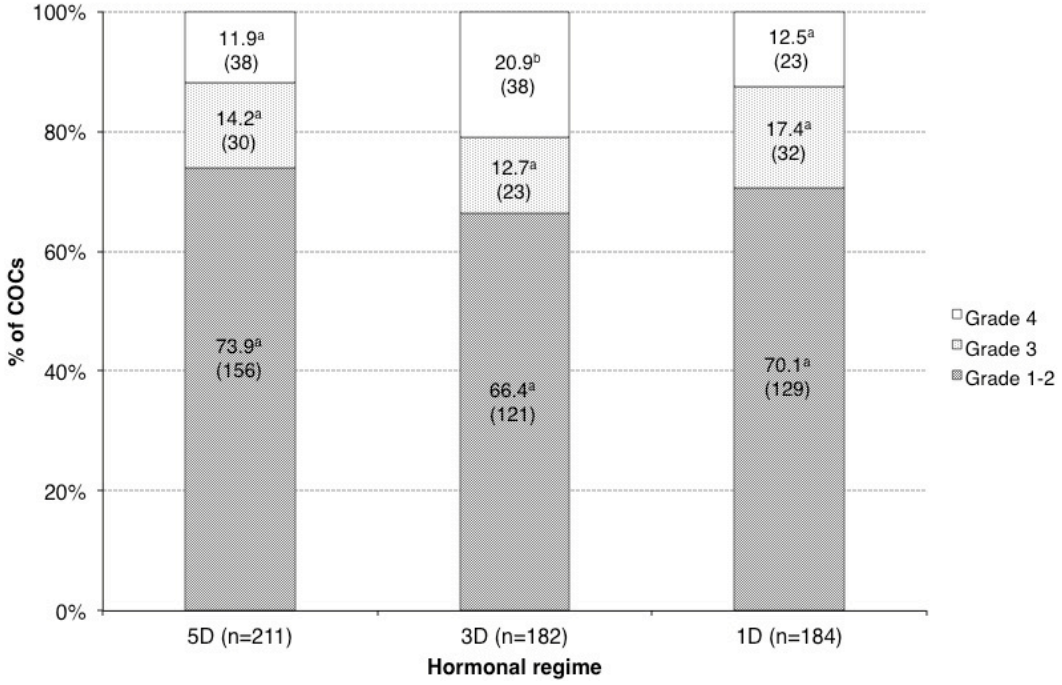

Figure 2. Distribution of various quality grades of *cumulus*-oocyte complexes (COCs) recovered from Canindé goats receiving different hormonal ovarian stimulation regimes.
[a, b]Denotes that there were significant differences ($P < 0.05$) between treatments within the same grade of COCs.

Discussion

The use of LOR, followed by IVM and *in vitro* fertilization (IVF) offers the potential of producing offspring from genetically important individuals when traditional multiple ovulation and embryo transfer procedures are not suitable (prepubertal, pregnant, puerperal animals, animals with uterine malformations and adhesions). In small ruminants, previous studies have shown that the number of oocytes recovered in one session can be increased when the donor females are hormonally stimulated (Morton *et al*., 2005). However, these hormonal treatments are still not well established in goats, and no information was found in the literature for naturalized goat breeds of Brazil. In the present study, it was demonstrated that hormonal ovarian stimulation treatments followed by LOR and IVM of the oocytes recovered can be used to preserve valuable genetic material from naturalized goats. To the best of our current knowledge, this is the first paper to report the reduction-to-practice of the application of the LOR platform to preserve endangered goat breeds.

The oocyte yields obtained in the present study using LOR after administration of the gonadotrophin treatments were similar to those previously described using single or multiple dose regimens. These studies (as in the current study) have reported outputs from 12 to 20 follicles and 8 to 15 COCs per donor (Pierson *et al*., 2004; Gibbons *et al*., 2007; Abdullah *et al*., 2008).

In studies regarding the use of LOR in goats, various researchers have reported an oocyte recovery rate of between 25 and 70% (Graff *et al*., 2000; Gibbons *et al*., 2007; Abdullah *et al*., 2008). Current results are similar to those obtained by Baldassarre *et al*. (2003), who reported recovery rates above 80%. LOR is a technique that has the advantage of being repeatable; however, its success can be strongly influenced by the operator's experience (Morton *et al*., 2005). In addition, the interval between the end of hormonal treatment and LOR may affect the recovery rate and oocyte quality (Abdullah *et al*., 2008).

In the present study, the quality of the COCs was affected in the 3D group (greater frequency of grade 4). The treatment regimen of the 3D group was based on earlier experiments performed by our group with naturalized goats (unpublished results). However, the long interval (24 h) between injections of FSH apparently impaired follicular development and the subsequent morphological quality of the COCs aspirated from these follicles.

The developmental potential of an embryo thus depends on the developmental potential of the oocyte from which it originates. The process of oocyte maturation is critical for the efficient application of biotechnologies such as IVEP and mammalian cloning. However, the overall efficiency of IVM remains low, as oocytes matured *in vitro* have lower developmental competence than oocytes matured *in vivo* (Lonergan *et al*., 2003).

The maturation rates obtained in this study for the two best treatments (5D and 1D) were similar to those reported by other studies (Graff *et al*., 2000), but not as successful as in recent reports in the literature for goats (Baldassarre *et al*., 2007; Abdullah *et al*., 2008; Leoni *et al*., 2009). Therefore, additional studies have to be undertaken in the laboratory to improve IVM rates and subsequent *in vitro* production of goat embryos.

It has already been reported that FSH requires the use of multiple doses due to its shorter half-life when compared to eCG (Monniaux *et al*., 1983). Thus, in despite of the use of the same amount of FSH in both treatments (5D and 3D), it was hypothesized that the intervals between the injections of FSH were too long in the 3D group. In this case, it is possible that inadequate gonadrotropin stimulation could decrease the oocyte's meiotic competence in recovered COCs.

Although the maturation rates from the 5D and 1D treatments were similar, there are some practical differences. eCG is less expensive and requires less labor input as it requires only a single injection.

In conclusion, taking into account the main performance results of the three treatments, it is advisable to use the 5D regime in future Canindé breed preservation programs based on laparoscopy technology as a means to recover oocytes. However, further experiments are necessary to verify both IVF and blastocyst development rates of matured oocytes obtained from these treatments.

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