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Original Research Article

Assessment of the reproductive parameters, laparoscopic oocyte recovery and the first embryos produced *in vitro* from endangered Canindé goats (*Capra hircus*)

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ABSTRACT

The Canindé breed of goats (*Capra hircus*) is currently endangered. The aims of this study were to characterize the estrus behavior, ovulatory responses and progesterone profiles, and to evaluate the *in vitro* embryo production (IVP) in this breed. In Experiment 1, ten nulliparous and seven pluriparous females received medroxyprogesterone acetate (MAP)-containing sponges (60 mg) plus 75 μg D-cloprostenol for estrus synchronization and their reproductive parameters were evaluated. In Experiment 2, oocytes obtained by laparascopy from hormonally stimulated females ($n = 15$) were used for IVP. There was no difference ($p > 0.05$) between nulliparous and pluriparous goats in terms of estrus response (40.0% *vs.* 85.7%), time from progestagen sponge removal to the onset of estrus (62.0 ± 15.5 *vs.* 50.7 ± 19.2 h; mean ± SEM), duration of estrus (25.0 ± 16.1 *vs.* 30.0 ± 15.1 h), percentage of ovulating animals (60.0% *vs.* 85.7%), number of ovulations (1.2 ± 0.4 *vs.* 1.3 ± 0.8), and diameter of the preovulatory follicle (5.8 ± 0.5 *vs.* 6.1 ± 0.3 mm). Progesterone concentrations were also similar ($p > 0.05$) in both groups. During laparoscopic recovery, there were average 12.2 aspirated follicles and 9.1 oocytes per goat, resulting in a high recovery rate (74.3%, 182/245). A total of 78 embryos were produced (51.0%). The mean number of cells in the blastocysts at day 7 of *in vitro* culture was 170.3 ± 12.5. In conclusion, nulliparous and pluriparous Canindé goats exhibited similar reproductive profiles. It was possible to produce embryos *in vitro*, allowing the instigation of an embryo bank for preservation of this breed.

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1. Introduction

When Europeans discovered Brazil, approximately 500 years ago, the Portuguese settlers brought the first ruminants to the country. Since then, these animals formed breeds that are called "naturalized" and for centuries contributed to the country's livestock production. Over the years, natural selection occurred and these breeds developed morphological and physiological characteristics specific for Brazilian environmental conditions. In the early 20th century, imported commercial breeds have gradually begun replacing the naturalized breeds to such an extent that the latter are now in danger of extinction [1]. The endangered Canindé goat, which is in this category of naturalized breeds, is found mainly in Northeastern Brazil. These animals are frequently raised in an extensive manner, receiving almost no human handling. The number of registered Canindé goats in the Brazilian Goats Breeders Association is around 200. However, it is possible to estimate that their total number is under 3000 animals. Studies on Canindé goats utilizing molecular markers are under way [1,2]. Greater sustainability of this breed may be vitally important for families that live on a subsistence basis by providing both milk and meat, which is possible due mainly to the breed's adaptation to dry environment. Therefore, efforts to preserve Canindé goats are important so that their genetic material is not permanently lost; however, the available literature regarding the reproductive physiology of this breed is scarce. Hence, it is necessary to better understand their reproductive biology and to determine the response of these animals when subjected to reproductive biotechnologies such as *in vitro* embryo production (IVP).

The viability rate of IVP embryos is lower than that of their *in vivo* counterparts generated by multiple ovulation and embryo transfer (MOET) procedures [3]. Moreover, the MOET embryos have a greater tolerance of cryopreservation than IVP embryos [4]. In small ruminants, *in vivo* embryo recovery requires surgical procedures that impair repeated embryo production from individual donors. In addition, some goats do not respond to superovulatory treatments but are still eligible for IVP. Therefore, both technologies have their advantages and disadvantages, but could be employed for the conservation of endangered species, as reviewed by Souza et al. [5]. The use of IVP to preserve an endangered breed requires the retrieval of oocytes *in situ*, since obviously such animals are not slaughtered very often. To recover high numbers of oocytes, the goats are pre-treated with a gonadotropin. Current treatments mostly consist of multi-injection follicle-stimulating hormone (FSH) regimes and the protocols are highly labor intensive and stressful to the animals because of excessive handling. For these reasons, there is a demand for simplified protocols [6]. Therefore, the one-shot regimen, in which a combination of FSH and equine chorionic gonadotropin (eCG) is given as a single treatment administered ~36 h prior to laparoscopic ovum pick up (LOPU) is a preferred option [7] that is less expensive and requires less labor input. Recently, our group has demonstrated that it is possible to use both protocols (five injections of FSH or a single dose of eCG and FSH) to stimulate antral follicular development in Canindé goats [8]. However, in that study, the oocytes were utilized for *in vitro* maturation (IVM) and ensuing molecular analyses, and so it was not possible to produce embryos *in vitro*.

Thus, the first objective of the present study was to characterize and compare the estrus, ovulatory responses and progesterone (P4) profiles of the estrous cycle in nulliparous and pluriparous Canindé goats. To avoid the residual effects of an exogenous gonadotropin, only a progestagen treatment combined with an injection of luteolysin was used for estrus synchronization. Additionally, we aimed to evaluate the efficacy of a simplified stimulatory protocol (a single dose of FSH and eCG) to yield good quality oocytes for recovery as well as to assess maturation, fertilization and culture systems for producing caprine embryos *in vitro*.

2. Materials and methods

2.1. Experimental animals and chemicals

This study was conducted in the Laboratory of Physiology and Control of Reproduction (LFCR; School of Veterinary Medicine, Ceará State University), Fortaleza, CE, Brazil (latitude 3°47′38″ S and longitude 38°33′29″ W). Female goats reared at this latitude are usually non-seasonal breeders, exhibiting recurrent estrous cycles throughout the year. Six months before the beginning of this study, goats from a herd raised in an extensive system with almost no human contact or handling had been brought to our lab. All of the animals were maintained in a semi-intensive system, receiving Tifton (*Cynodon dactylon*) hay and daily access (4 h) to the pasture of this grass variety. In addition, the animals' diets were supplemented with 0.2 kg/day of commercial concentrate (a minimum of 20% crude protein) and they had free access to water and mineralized salt licks. All of the females were clinically and ultrasongraphically examined (Falco 100; Pie Medical, Maastricht, Netherlands) to detect pregnancy or any abnormalities of the reproductive tract. The use and care of the animals in this study were approved by the Animal Ethics Committee of Ceará State University (CEUA/UECE, n° 09144595-7/50). All of the trials were conducted in accordance with the guidelines for animal care [9]. Unless indicated otherwise, all of the chemicals and reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Experimental procedures

2.2.1. Estrus and ovulatory responses (Experiment 1)

Seven pluriparous and ten nulliparous, sexually mature Canindé goats were used in this experiment (weight: 27.6 ± 3.8 kg; body condition score, BCS, 1–5 scale [10]: 3.0 ± 0.2; age: 1–4 years). All of the does received intravaginal sponges containing 60 mg of medroxyprogesterone acetate (MAP; Progespon, Syntex, Buenos Aires, Argentina) for 6 days and 75 μg of D-cloprostenol (Prolise; Pfizer Animal Health, São Paulo, Brazil) 24 h before sponge removal. After sponge removal, estrus was monitored by observing the reactions of the females to the presence of four fertile Canindé bucks every 4 h for 96 h; goats were recorded as being in estrus if they accepted being mounted. After that, estrus monitoring was performed twice daily until either the subsequent estrus or for the following 21 days.

Transrectal ovarian ultrasonographic examinations, using a Falcon 100 scanner (Pie-Medical, Maastricht, The Netherlands), were performed on all animals every 12 h after sponge removal until ovulation was detected or up to 120 h later by the same operator. Six days after estrus, another ultrasonographic examination was performed to confirm the number and side of ovulations. A 6.0/8.0-MHz linear-array transducer (60 mm; reference number: 410054) was inserted into a stiffener for external manipulation in the rectum. The animals were examined in a standing position. Fecal pellets were manually removed and carboxymethylcellulose gel (15–20 ml) was administered with a syringe into the rectum. The procedure used to locate the ovaries was previously described [11]. The number, diameter and position of all ovarian follicles ≥3 mm in diameter were recorded. The day of ovulation was defined as the day of disappearance of the largest antral follicle (>5 mm) that had been identified before.

Blood samples were collected from all of the goats by jugular venipuncture using 4-ml tubes containing EDTA (BD Vacutainer, Becton Dickinson and Company, Holdrege, NE, USA) for determination of the plasma P4 concentration. Samples were obtained daily (08:00 am) after sponge removal (day 6) until the next estrus or up to 21 days after the first estrus (approximately day 31). The tubes were immediately placed on ice until centrifugation at 2000 × *g* for 15 min. Plasma was aliquoted and stored at −20 °C until the hormone assay. Progesterone concentrations were measured using a commercial, solid-phase radioimmunoassay kit (Coat-A-Count; DPC, Diagnostic Products Corporation, Los Angeles, CA, USA) according to the manufacturer's instructions. The mean intra- and inter-assay coefficients of variation were 8.8% and 9.7%, respectively, and the analytical detection limit was 0.08 ng/ml.

The percentage of ovulating goats as well as the location and the number of corpora lutea (CLs) were additionally determined by laparoscopy, six days after synchronized estrus, immediately after the last ultrasonographic examination. The females were deprived of food and water for 24 h prior to laparoscopy. Anesthesia was done using 0.3 mg/kg body weight (b.w.) of xylazine hydrochloride (Dorcipec; Vallée, Montes Claros, Brazil) and 0.04 mg/kg b.w. of atropine (Atropina 1%; Fagra, Mairiporã, Brazil). In addition, local anesthesia with 2% chlorhydrate lidocaine (Anestésico L Pearson, Eurofarma, São Paulo, Brazil) was applied into the trocar puncture sites. The donor goats were restrained on a standard laparoscopy table. The procedure utilized a 5-mm laparoscope (Karl Storz Endoscopes GmbH & Co., Tuttlingen, Germany) attached to a video system. The laparoscope was inserted into the abdominal cavity through a trocar (cranial to the udder and to the left side of the midline). An atraumatic grasper was inserted into the right side of the abdomen to hold the ovary, making it possible to locate and count corpora lutea. Finally, the trocar wounds were treated with a local antibiotic/healing solution (Terra-cortril spray; Pfizer Animal Health).

2.2.2. *In vitro embryo production and vitrification (Experiment 2)*

Nine nulliparous and six pluriparous Canindé goats aged 2–4 years were used as oocyte donors. Five goats were used twice and 10 goats only once. All of the females received intravaginal sponges containing 60 mg of MAP for 11 days and 75 μg of D-cloprostenol at day 8 of the progestagen treatment. The goats received a single dose of 70 mg of porcine FSH (Folltropin-V; Vetrepharm, Ontario, Canada) plus 200 IU of eCG (Novormon; Syntex) 36 h before sponge removal to stimulate antral follicle growth. Laparoscopic ovum pick up (LOPU) was performed on the day of sponge removal. Animals were deprived of food for 36 h and water for 24 h prior to laparoscopy. Anesthesia was induced by administration of 20 mg/kg b.w. of thiopental (Tiopentax 2.5%; Cristália, São Paulo, Brazil) i.v. and maintained by continuous infusion of 3% isoflurane (Isofrine; Cristália), using an inhalational system with medical oxygen (HB Hospitalar, São Paulo, Brazil). Local anesthesia was applied as described earlier.

LOPU was performed immediately after sponge removal. The ovary was held by the grasper, and ovarian follicles were individually aspirated using a 22-gauge needle connected to an aspiration and a flushing system (WTA, Cravinhos, Brazil). The vacuum pressure was set at −30 mmHg, generating a fluid flow of 7–7.5 ml/min. All follicles larger than 2 mm were aspirated, and all small (<3 mm), medium (3–4 mm) and large (>4 mm) follicles were counted. Follicle measurements were performed with the fenestrated grasping forceps that had a 4-mm scale bar. The collection medium used was TCM 199 containing 10 mM HEPES, 0.022 μg/ml of sodium pyruvate, 10,000 IU of penicillin, 10,000 μg/ml of streptomycin sulfate, 25 μg/ml of amphotericin B, 10% fetal calf serum (FCS), and 20 IU/ml of heparin sulfate. Once the LOPU was completed, each ovary was gently flushed with a heparinized saline solution (25 IU/ml) at 37 °C to prevent the formation of adhesions. Finally, the trocar orifices were treated locally with an antibiotic solution.

The quality of cumulus-oophorus complexes (COCs) was assessed by stereomicroscopy (SMZ 800; Nikon, Tokyo, Japan), with different grades [8] as follows: grade 1 – multilayered compact cumulus and finely granulated oocyte cytoplasm; grade 2 – one to three layers of cumulus cells and finely granulated oocyte cytoplasm; grade 3 – incomplete cellular investment or heterogeneous oocyte cytoplasm; grade 4 – oocyte with abnormal shape and heterogeneous oocyte cytoplasm. COCs classified as grades 1, 2 and 3 were considered good quality oocytes that were suitable for IVM; the remaining oocytes, classified as grade 4, were discarded. The COCs were washed four times and transferred to maturation medium consisting of TCM199 supplemented with 0.022 μg/ml of sodium pyruvate, 10,000 IU of penicillin, 10,000 μg/ml of streptomycin sulfate, 25 μg/ml of amphotericin B, 10% of fetal calf serum (FCS), 10 ng/ml of epidermal growth factor, 5 μg/ml of porcine FSH, 10 μg/ml of porcine luteinizing hormone, 1 μg/ml of 17β-estradiol, and 100 μM of cysteamine. The COCs were incubated for 24 h at 38.5 °C in a humidified atmosphere of 5% CO_2 in air.

Fresh semen was collected from Canindé bucks of confirmed fertility. Motile sperm was obtained by centrifugation (15 min at 900 × *g*) on a 2-ml Percoll discontinuous density gradient (45/90%). The viable spermatozoa were diluted in the appropriate volume of fertilization medium and added to each fertilization drop to achieve a final concentration of 2.0×10^6 spermatozoa/ml. At the conclusion of IVM, the COCs were transferred to fertilization medium drops [12]. The sperm and oocytes were co-incubated for 18 h at 38.5 °C in a humidified atmosphere of 5% CO_2 in air.

Table 1 – Reproductive parameters (mean ± SEM) of nulliparous and pluriparous Canindé goats subjected to estrus synchronization.

Variables	Nulliparous goats	Pluriparous goats
Estrus response (%)[a]	40.0 (4/10)	85.7 (6/7)
Time to the onset of estrus (h)[b]	62.0 ± 7.7	50.7 ± 7.8
Duration of estrus (h)[c]	25.0 ± 8.1	30.0 ± 6.2
Interval from sponge removal to ovulation (h)	106.0 ± 4.8	110.0 ± 4.8
Interval from the onset of estrus to ovulation (h)	49.0 ± 5.0	59.3 ± 7.4
Percentage of ovulating goats (%)[d]	60.0 (6/10)	85.7 (6/7)
Number of ovulations per goat	1.2 ± 0.2	1.3 ± 0.3
Diameter of the preovulatory follicle (mm)	5.8 ± 0.2	6.1 ± 0.1
Ovulation on right ovary (%)	71.4 (5/7)	50.0 (4/8)
Ovulation on left ovary (%)	28.6 (2/7)	50.0 (4/8)

No differences were found between nulliparous and pluriparous goats in the examined parameters.
[a] Number of goats in estrus/number of treated females 100×.
[b] Interval from sponge removal to first acceptance of mounting.
[c] Interval from the first to last acceptance of mounting.
[d] The number of goats with confirmed ovulation/the total number of goats evaluated by ultrasonography 100×.

After fertilization, the COCs were denuded by vortexing. The presumptive zygotes were washed four times in the culture medium containing modified synthetic oviductal fluid with amino acids (SOFaa) medium [13] supplemented with 0.4% of bovine serum albumine (BSA) and 2% FCS to remove the spermatozoa, transferred to 50-μl drops of culture medium and covered with mineral oil. The presumptive zygotes were incubated for 7 days at 38.5 °C in a humidified atmosphere of 5% CO_2 in air. At 48 h post-insemination, 50% of the medium was replaced with fresh medium. Cleavage was evaluated on day 2 of the culture, and the number of blastocysts was counted on day 7 post-*in vitro* fertilization (IVF). The quality and developmental stage of embryos that reached blastocyst (early blastocyst, blastocyst, expanded blastocyst or hatched/hatching blastocyst) was determined according to the Manual of the International Embryo Transfer Society (IETS). Additionally, the quality of the expanded blastocysts was assessed using a simple staining technique. Briefly, twelve embryos were fixed with 4% paraformaldehyde (F1635) in phosphate buffered saline (PBS) for approximately 15 min at 25 °C. For cell counts, the blastocysts were incubated in 10 μg/ml bisbenzimide (Hoechst 33342, B2261) for 15 min at 25 °C. Thereafter, the samples were placed in a glycerol droplet on a glass slide and carefully covered with a coverslip. The samples were examined under a fluorescence microscope (TE200; Nikon, Japan) equipped with a UV filter. Other blastocysts and hatched embryos were vitrified using a method described by Vajta et al. [14].

2.3. *Variables and statistical analysis*

The variables determined in Experiment 1 are listed in Table 1. Comparisons were made between the nulliparous and pluriparous animals using Mann–Whitney–Wilcoxon test or Fisher's exact test when appropriate (Prism 5.0a, GraphPad Software; San Diego, CA, USA, 2007). The following variables were determined in Experiment 2: total numbers of detected follicles; numbers of aspirated follicles; numbers of small (<3 mm), medium (3–4 mm) and large (>4 mm) follicles; mean numbers of aspirated follicles and oocytes recovered per goat in all of the sessions; oocyte recovery rate (%) (number of recovered oocytes/number of aspirated follicles × 100); oocyte quality at collection; cleavage rate (%) (percentage of cleaved oocytes 2 days post-IVF); blastocyst formation rate (%) (percentage of blastocysts at day 7 post-insemination (p.i.) relative to the total number of oocytes used for IVM or the number of cleaved oocytes on day 2 p.i.); embryo quality evaluated by stage classification on day 7 p.i.; and mean numbers of cells per expanded blastocyst. All of the results are given as the mean values ± SEM.

3. Results

3.1. *Estrus, ovulatory responses and progesterone profiles (Experiment 1)*

Of the 17 synchronized goats, 10 showed signs of estrus followed by ovulation, and two ovulated without displaying estrus. Four goats showed neither estrus or ovulation. One goat was in estrus 108 h after sponge removal and was excluded from analysis. There was no significant difference ($p > 0.05$) between the nulliparous and pluriparous females for any of the end points analyzed in this experiment (Table 1). One nulliparous goat had two ovulations including the ovulation of the second follicle of 5.5 mm in diameter, and one pluriparous goat had three ovulations including the ovulations of the second (5.7 mm) and third largest follicle (5.5 mm in diameter). None of the goats exhibited estrus while the sponges were in place.

The percentage of goats with ovulation confirmed by ultrasonographic examinations or by laparoscopy was the same. During the laparoscopic examination of the ovaries, 2/12 pluriparous goats that ovulated (16.7%) had only abnormal corpora lutea (CL; avascular and pale, indicative of premature luteolysis), and one animal had one normal and one abnormal CL. Both animals with short-lived CL returned to estrus 4–5 days after the first estrus. Two pluriparous and two nulliparous goats returned to estrus 20–21 days after the synchronized estrus. Plasma P4 concentrations did not differ ($p > 0.05$) between the nulliparous and pluriparous females. Mean P4 concentration was 0.5 ± 0.1 ng/ml in metestrus (mean concentration from the day of estrus up to the first analysis greater

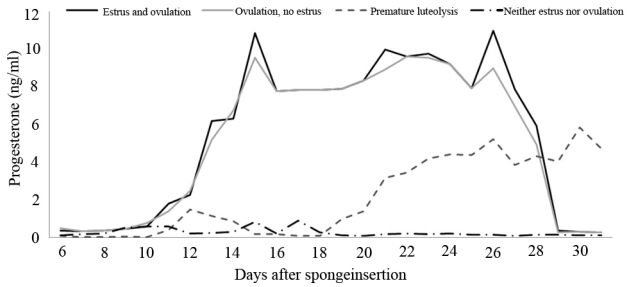

Fig. 1 – Plasma progesterone concentrations in four Canindé goats that exhibited different behavioral and ovulatory responses after estrus synchronization.

than 1 ng/ml – on average from day 2 to 4 after sponge removal) and 5.2 ± 0.2 ng/ml in diestrus (from the end of metestrus up to the last day before luteolysis – on average from day 5 to 21 after sponge removal). Progesterone concentrations in four individual goats differing in estrus responses are shown in Fig. 1.

3.2. *Retrieval of cumulus oophorus complexes (COCs) obtained by LOPU and in vitro embryo production (Experiment 2)*

In four replications (four LOPU sessions), 310 follicles were visualized and 245 were aspirated, 130 from the left ovary and 115 from the right ovary. An average of 12.2 aspirated follicles and 9.1 recovered oocytes were obtained from each donor goat in all of the sessions, resulting in an overall oocyte recovery rate of 74.3% (182/245). Seventy-seven oocytes (31.4%) were aspirated from small follicles, 88 (35.9%) from medium-sized follicles, and 80 (32.7%) from large ovarian follicles. A majority of the oocytes collected was of good quality [24 (grade 1), 124 (grade 2) and 10 (grade 3)], and only 24 were discarded (grade 4). A total of 153 COCs were subsequently used for IVP.

The mean cleavage rate was 58.8% (90/153). A total of 78 embryos were produced, resulting in 86.7% (relative to the numbers of cleaved oocytes) or a 51.0% blastocyst formation rate (relative to the total number of oocytes used for IVM; Fig. 2A). The distribution of blastocysts was as follows: early blastocysts (12.8%), blastocysts (35.9%), expanded blastocysts (16.7%) and hatched blastocyst (34.6%). Twelve of the expanded blastocyst (Fig. 2B and C) was fixed and their nuclei counted; the mean blastocyst cell number was 170.3 ± 12.5. The other blastocysts and hatched embryos were vitrified, thus forming the embryo bank for the Canindé breed. Early blastocysts were discarded since they could be arrested blastocysts that are not suitable for subsequent transfers.

4. Discussion

The present study is the first report of the successful *in vitro* embryo production in the endangered Canindé goat. It is also the first description of the reproductive parameters recorded during the estrous cycle (estrus, ovulation and P4 profiles) in this breed. Because no statistically significant difference between the nulliparous and pluriparous animals was detected for any of the reproductive variables analyzed, the averaged values were assessed. The mean percentage of goats showing estrus was 58.8%. Although not significantly different, a greater percentage of pluriparous goats (85.7%) entered estrus compared with the nulliparous does (40.0%). This numerical difference could be due to a greater degree of maturation of the reproductive hormonal axis in pluriparous goats. An earlier study has shown that different hormonal profiles detected in nulliparous and pluriparous goats may contribute to the lower reproductive performance of the nulliparous females [15]. The duration of estrus in the goats of the present study averaged 28 h, similar to the 25-h estrus observed in the Alpine breed [16] or the 31-h estrus in the Saanen breed [17], but shorter than the 59 h reported for Matou goats [18].

The interval from the removal of intravaginal progestagen-releasing sponges to the onset of estrus was on average 55 h (ranging from 32 to 88 h), which was similar to the 53-h interval reported for the Boer breed [19]. The time to the onset of estrus in goats is highly variable, as shown in several studies that had employed hormonal synchronization treatments [20,21]. In a study using ovariectomized goats, Freitas et al. [22] have demonstrated that treatments based on progestagen administration were unlikely to improve the synchrony of the estrus onset, which is supportive of the notion that the disperse onset of estrus is an inherent feature in goats. Injecting eCG is known to reduce the interval between the removal of progestagen source and the onset of estrus, and to affect the duration of estrus [23]. However, in the present study, eCG was omitted to obtain the response that was not confounded by an effect of exogenous gonadotropin.

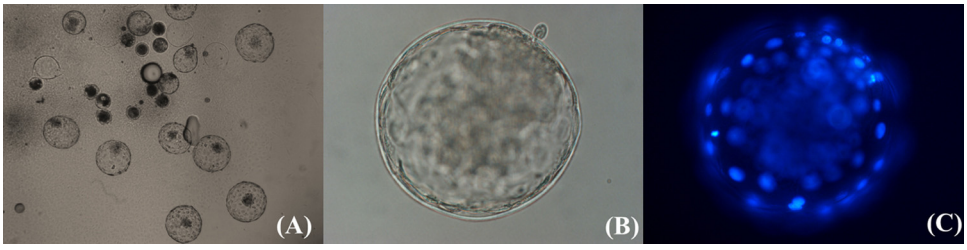

Fig. 2 – *In vitro* produced Canindé goat embryos cultured in modified synthetic oviductal fluid with amino acids (SOFaa) medium. (A) Blastocysts on day 7 of *in vitro* culture (100×); (B) expanded blastocyst on day 7 (400×); and (C) the cell nuclei of an expanded blastocyst on day 7 of *in vitro* culture viewed under UV light after nuclear staining (400×).

In the present study, the percentage of ovulating goats was on average 70.5%. Although the difference was not significant, a greater proportion of pluriparous goats (85.7%) ovulated compared with the nulliparous animals (60.0%). As with the signs of estrus observed, this difference could be attributed to a more advanced development of the hormonal reproductive system of the pluriparous goats. Simões et al. [24] reported significant differences in the number and timing of the ovulations between nulliparous and pluriparous Serrana goats. The mean number of ovulations per goat observed in the present study (1.3) was similar to that observed in the Alpine (1.4), Saanen (1.2) [25] and Nubian breeds (1.4) [26]; none of those studies used additional gonadotropin treatments. The number of ovulations is a good predictor of prolificacy. Thus, it is reasonable to assume that the prolificacy of the Canindé breed is similar to that of the well-known and most widespread breeds of goats.

The slightly higher ovulation frequency on the right ovary (60.0% *vs.* 40.0%) noted in this study is in agreement with earlier findings in dairy goats [27]. Chavez et al. [28] observed this phenomenon in rats when they discovered the asymmetry of the impulses carried to ovaries by the right and left vagal nerve. The uni- or bilateral resection of the vagal nerve produced different results *in situ*; ovulation rate in unilaterally ovariectomized rats was lower in the left than the right ovary (42% *vs.* 84%). The authors concluded that, in the small rodent model used, regulatory compensatory systems are more likely to occur in the right ovary than in the left one.

The percentages of ovulating goats detected by ultrasonography or laparoscopy were the same, which confirms the reliability of the ultrasonography to determine this parameter, and the less invasive ultrasonographic technique is a preferred option. However, the laparoscopic examination of the ovaries revealed the presence of abnormal CLs undergoing premature luteolysis in 2 out of 12 does (16.7%) but at the time of examination, these structures could not be identified with ultrasonography as inadequate CLs. The P4 profile of one female with abnormal CLs only is shown in Fig. 1 (premature luteolysis). Interestingly, this female showed signs of estrus on day 8 (two days after sponge removal), with an estrus that lasted up to day 10, and again on day 15 (five days after the end of the first estrus). Both goats with apparently normal CL had greater concentrations of P4 on day 15 compared to the animal with premature luteolysis. Two does ovulated without displaying signs of estrus, which is observed occasionally in goats [19,29]. Notably, from the day of sponge removal to the last day of the next cycle after the synchronized estrus, these females had P4 profiles similar to those in the animals that exhibited estrus, suggesting that the silent ovulation was not related to low circulating levels of progesterone.

Circulating P4 concentrations remained at basal levels throughout estrus, as previously observed [30,31]. In Markhoz goats, P4 levels gradually increased during metestrus and reached maximum values on day 12 during the luteal phase, ranging from 5 to 12 ng/ml [31]. In Damascus goats, P4 concentrations during the luteal phase ranged from 2.6 to 5.4 ng/ml [30], similar to our findings in the present study. Besides the breed-related differences, variations in P4 concentrations between our current and those earlier studies could have been due to the accuracy of P4 assays among laboratories.

In an earlier study, there were no significant differences between the ovarian stimulation protocol using both eCG and FSH compared with the standard treatment with several doses of FSH in terms of the number of ova and embryos recovered from superovulated sheep [6]. Moreover, the simplified stimulation treatment appeared to be well suited for the Canindé breed because it resulted in the aspiration of an average 12.2 follicles and recovery of ~9.1 oocytes per donor animal, similar to the results previously obtained from other goat breeds after the hormonal treatment and LOPU [32,33]. In a previous study using a similar ovarian stimulatory protocol in the same breed [8] but with the oocytes denuded before fertilization, we obtained an oocyte maturation rate of 46%; the cleavage rate was not assessed [8]. We believe that cumulus cells play an important role during fertilization because their presence resulted in significantly improved development of zygotes to the blastocyst stage [34]. Thus, in the current study, the cumulus cells remained attached to the oocytes during fertilization and, therefore, it was not possible to assess the maturation rate after IVM. However, it is reasonable to assume that the maturation rate was higher now because the cleavage rate was higher and only matured oocytes can be fertilized. The overall cleavage rate was 58.8%, lower than the 82% rate [35] and slightly greater than the 51% rate [32] obtained in studies that also used the oocytes recovered by LOPU. We believe that it may be possible to improve the rate in subsequent studies by ameliorating IVM and IVF conditions for LOPU oocytes' requirements.

The blastocyst formation rate relative to the number of oocytes that cleaved (86.7%) or to the total number of oocytes used for IVM (51.0%) was similar to or higher than those in other studies in goats [12,35]. The developmental potential of embryos in terms of blastocyst yields depends on the developmental potential of the oocyte from which it originates (i.e., its intrinsic quality) but also on the conditions to which they are subjected [36]. Moreover, the mean number of cells in the blastocysts observed in this study was similar to that found in other studies in goats [32,37], regardless of the differences in IVC conditions. Most of the hatching and hatched embryos survived cryopreservation, exhibiting higher survival rates than those vitrified at earlier stages of development [38].

In summary, the present study will serve as a basis for upcoming genetic investigations in the Canindé breed. This may be an important tool for their conservation. Moreover, it was possible for the first time to produce embryos *in vitro* and to start forming a bank of frozen embryos for this breed from oocytes obtained by follicular puncture using laparoscopy. Further studies are necessary to evaluate the *in vivo* viability of the vitrified IVP goat embryos.

Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the article reported.

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