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Survival, growth, and hormone production *in vitro* from preantral follicles of crossbred female goats (Saanen × Anglo-Nubian)

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Abstract

The present study aimed to evaluate the survival, growth, antrum formation, oocyte extrusion, and hormone production during *in vitro* culture of caprine preantral follicles isolated from pure-breed (Saanen) or crossbreed (F1 generation: ½ Saanen + ½ Anglo-Nubian) goats. Secondary follicles (diameter: 150-250 µm) from Saanen or crossbreed (Saanen × Anglo-Nubian) goats were isolated from the ovarian cortex by microdissection and cultured *in vitro* for 18 days in α-modified minimum essential medium (α-MEM+) supplemented with vascular endothelial growth factor and increasing concentrations of follicle-stimulating hormone. Every six days follicular morphology, growth, antrum formation, and follicular extrusion were evaluated. In addition, on days 2, 6, 12, and 18 of culture the medium samples were collected and stored at -20°C for further measurement of estradiol and progesterone. The follicular survival, antrum formation, and oocyte extrusion were analyzed by the Chi square test. Follicular diameter and hormone assays were compared using the Kruskal-Wallis test. Survival rates, growth, antral follicle formation, and oocyte extrusion of preantral follicles cultured *in vitro* were similar between the different genetic groups. The production of estradiol and progesterone indicated the maintenance of cell viability throughout the culture. In conclusion, preantral follicles from pure-breed or crossbreed goats can be used with the same efficiency for *in vitro* culture of isolated caprine preantral follicles.

Keywords: goat, oocyte, preantral follicles, pure-breed.

Introduction

Goats are of high economic importance in several countries for their contribution in the production of meat, milk, and skin (Paula *et al*., 2008). In Brazil, goats are concentrated in the northeast (more than 93% of the stock), a region with varying agro-climatic characteristics and large semiarid areas (Lôbo *et al*., 2010). The Saanen breed, as a more productive herd, was the first import of animals specialized in milk production in this region (Ferraz, 2007). Despite its high milk production capability, this breed lacks rusticity and, consequently, has low adaptability to conditions in the northeast, primarily because of the depigmented skin (Instituto Ambiental Brasil Sustentável - IABS, 2011). In this context, the Saanen and Anglo-Nubian breeds were crossed in an attempt to strengthen the rusticity. Anglo-Nubian goats have great rusticity, are of African origin, and adapt perfectly to the different regions in Brazil (IABS, 2011). However, this type of breeding might alter the reproductive behavior of the animals, therefore, understanding the physiology of the crossbreeds and the parents is necessary in order to adopt an appropriate reproductive management strategy.

Studies comparing the reproductive parameters between Saanen and Anglo-Nubian goats and their crossbreeds are scarce in literature, and few studies have performed such investigation to determine the age at puberty and estrous cycle length (Lopes Júnior *et al*., 2001, Cruz *et al*., 2003; Freitas *et al*., 2004). Ferraz *et al*. (2009) verified that although Saanen goats enter puberty earlier than Anglo-Nubian goats, their crossbreeds enter puberty at an intermediate age; however, there was no significant difference in the duration of estrus between the breeds (Ferraz *et al*., 2009). Regarding the ovarian follicular dynamics, only one study evaluated the *in vivo* development of antral follicles in Saanen and Anglo-Nubian goats (Cruz *et al*., 2005). However, no study has yet investigated the dynamics of preantral follicles in these breeds.

In vitro culture of ovarian follicles has emerged as a potential reproductive biotechnology method for producing large numbers of mature oocytes that are capable of fertilization (Demeestere *et al*., 2005). This bio technique aims to obtain mature oocytes from a

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population of preantral follicles found in large number within the mammalian ovary to produce embryos. Several studies have conducted preantral follicle culture and primarily investigated the effect of culture systems and medium supplements (Xu *et al*., 2006). Only a few studies have determined the effects inherent to the ovarian follicles from donor animals according to their age or reproductive cycle stage (Hirshfeld-Cytron *et al*., 2011). Since the cross between the two goat breeds has been intensively used to improve animal productivity and it has already been shown to have some effects on reproductive parameters, it can be assumed that the *in vitro* development of caprine preantral follicles is also influenced. The present study aimed to evaluate follicular development during the *in vitro* culture of caprine preantral follicles isolated from pure-breed (Saanen) or crossbreed (½ Saanen + ½ Anglo-Nubian, F1 generation) goats.

Material and Methods

All chemicals used were purchased from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO, USA). Ovaries (n = 26) from nine pure-breed (Saanen) and four crossbreed (½ Saanen + ½ Anglo-Nubian, F1 generation) cyclic goats (*Capra hircus*) were collected from a local slaughterhouse. The goats were aged between 14 and 38 months, with body score between 1.75 and 2.5 and weight between 33 and 39 kg. The breeding site is located between 3°53′49.9″ S and 38°34′32.5″ W at an altitude of 69 m in tropical climate. The animals were fed ad libitum a diet containing 70% elephant grass (*Pennisetum purpureum*) and 30% leucaena (*Leucaena leucocephala*), with 22.5% crude protein concentrate, water, and mineral salt. Immediately after slaughter, the ovaries were washed in 70% alcohol and minimum essential medium (MEM) supplemented with HEPES, penicillin (100 µg/ml), and streptomycin (100 µg/ml). The ovaries were submitted to a microdissection procedure to recover preantral follicles of >150 µm (Silva *et al*., 2010). Preantral follicles (n = 57 and n = 39 isolated from pure-breed and crossbreed ovaries, respectively) containing a visible oocyte surrounded by granulosa cells and with intact basement membrane were selected for *in vitro* culture. The follicles were cultured in 100 µl culture medium drops under mineral oil in petri dishes (60 × 15 mm; Corning, USA). Every other day 60 µl of the culture media were replaced with fresh medium, and the medium collected was stored at -20°C for subsequent hormonal measurements. The culture medium hereafter referred to as α-MEM^{+}, consisted of α-MEM (pH 7.2–7.4; Gibco, Invitrogen, Karlsruhe, Germany) supplemented with 3.0 mg/ml bovine serum albumin (BSA), 10 µg/ml insulin, 5.5 µg/ml transferrin, 5.0 ng/mL selenium, 2 mM glutamine, 2 mM hypoxanthine, 1 mg/ml bovine fetuin, 50 µg/ml ascorbic acid, along with 100 ng/ml vascular endothelial growth factor (VEGF) and recombinant human follicle-stimulating hormone (FSH; Nanocore, São Paulo, Brazil) in increasing concentrations (sequential FSH: day 0, 100 ng/ml; day 6, 500 ng/ml; day 12, 1,000 ng/ml). The culture conditions were 39°C in 5% CO_2 in air for 18 days. Oocyte viability was assessed using epifluorescence microscopy by using a marker for live (calcein-AM) or dead (ethidium homodimer-1) cells as previously described (Silva *et al*., 2010). Estradiol (E2) and progesterone (P4) secretions were determined by double antibody radioimmunoassay (RIA) by using MP kits (MP Biomedicals, Solon, USA). The lower detection limit and intra-assay coefficient of variation were 5 pg/ml and 2.5% for E2 and 0.01 ng/ml and 3.7% for P4, respectively. Antrum formation, oocyte extrusion, and viability were analyzed with the Chi square test. Data for growth rate and hormone assay did not show homoscedasticity and were compared using the non-parametric Kruskal-Wallis test. Results are expressed as mean ± standard error of means (SEM), and differences were considered to be significant when $P < 0.05$.

Results

After microdissection, preantral follicles isolated from the ovaries of pure-breed (n = 57) and crossbreed (n = 39) goats were recovered. After 18 days of culture, the percentages of antrum formation (Saanen: 40.82%; crossbreed: 45.45%) and oocyte extrusion (Saanen: 53.06%; crossbreed: 40.91%) were similar ($P > 0.05$). In addition, the growth rate (µm ± standard deviation) was not statistically different between the groups (Saanen: 19.60 ± 9.18; crossbreed: 18.02 ± 6.72). The viability assay is shown (Fig. 1A and 1B). The viability of oocytes at the end of the culture was similar between the groups tested (Saanen: 55.10%; crossbreed: 63.64%). A significant increase in the production of estradiol was observed from days 2 to 12, with no subsequent increase ($P > 0.05$) until the end of the culture in both groups. There was no significant difference between the groups in the E2 and P4 levels on any of the evaluated days of culture (Fig. 1C and 1D).

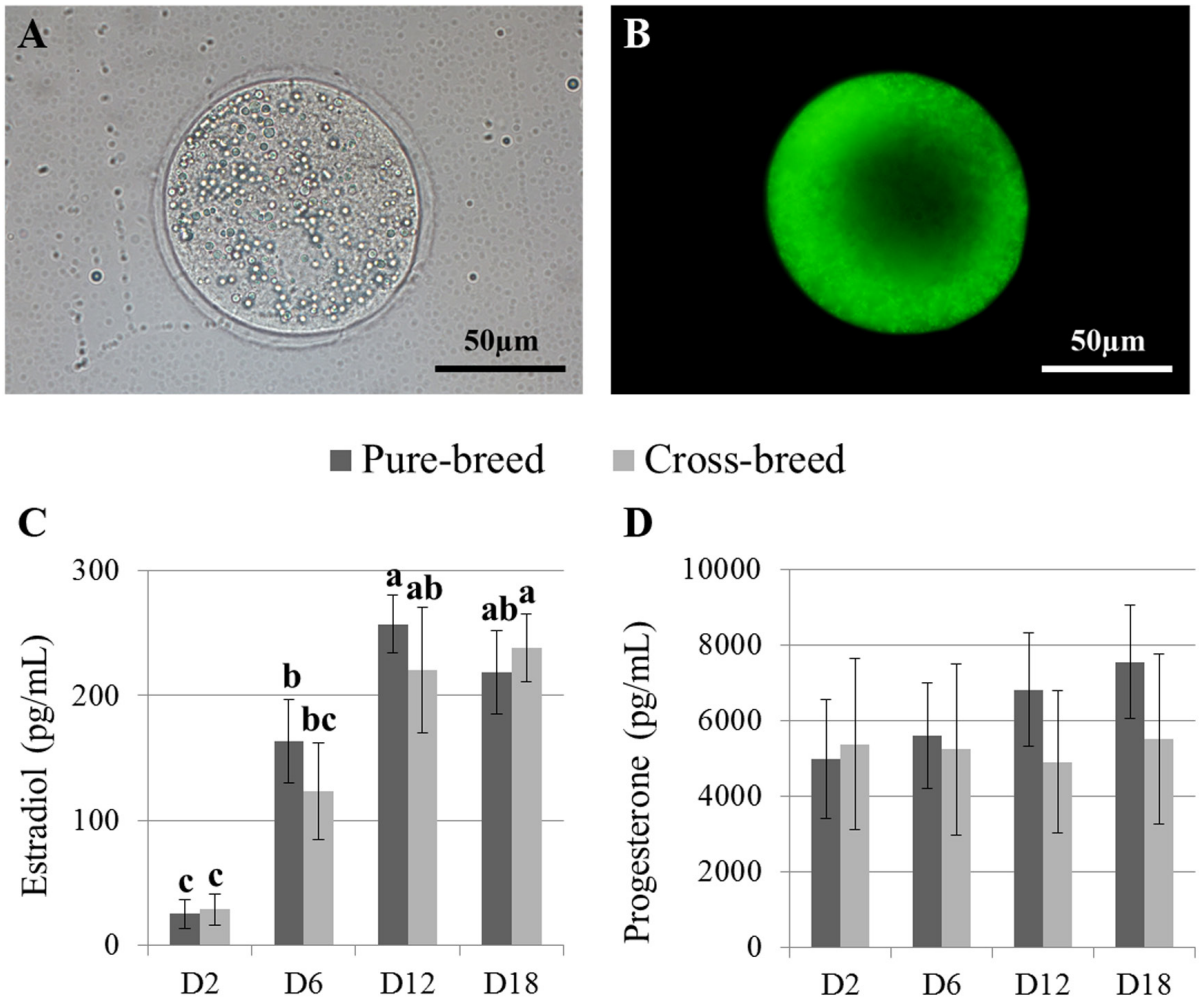

Figure 1. Viability of representative oocytes (A: oocyte visualization in bright field, B: characterization of a viable oocyte after staining with calcein-AM) and estradiol (C) and progesterone (D) production of secondary follicles grown *in vitro* from pure-breed (Saanen) or crossbreed (Saanen × Anglo-Nubian) goats. [abc]Comparison across days with the same treatment ($P < 0.05$). Media ± standard error of the mean (SEM).

Discussion

The present study compared, for the first time, the *in vitro* development of caprine preantral follicles isolated from pure-breed (Saanen) or crossbreed (F1 generation: ½ Saanen + ½ Anglo-Nubian) goats. In our study, the percentage of antrum formation, oocyte extrusion and oocyte viability were similar between the groups. In addition, there was no significant difference between the groups in the folicular growth rates and in the levels of E2 and P4 on any of the evaluated days of culture.

Few studies have compared the reproductive parameters of Saanen and Anglo-Nubian goats and their crossbreeds. Salmito-Vanderley (1999) compared the Saanen and Anglo-Nubian breeds raised under same conditions and found that age and weight at puberty were 293.2 and 165.9 days and 26.8 and 22.9 kg on average, respectively. Similar data were obtained by Ferraz *et al*. (2009). However, there was no difference in estrous behavior among the pure-breed goats (Saanen and Anglo-Nubian) and their crossbreeds (F1: ½ Saanen + ½ Anglo-Nubian) once puberty had begun or at the age after giving birth for the first time and the interval between births (Lôbo *et al*., 2005). More specifically, regarding ovarian follicular dynamics, Cruz *et al*. (2005) evaluated, for the first time, the follicular antral population and the dynamics of these follicles in Saanen and Anglo-Nubian goats. In this study, Anglo-Nubian and Saanen goats raised in a tropical climate were found to have a period of anestrus and ovulatory inactivity in the summer; however, during this period, the ovaries remained active with the number and interval between the start of a wave and the beginning of the next similar wave becoming regular, i.e., from 4 to 6 days. During this period, antral follicles continued to grow to the equivalent size of pre-ovulatory follicles with no significant differences between the 2 breeds. These data show that, after entering puberty, there are no

differences between the reproductive parameters among these breeds. In our study we used only adult animals that were already cycling, and management conditions were standardized (animals were from the same farm) to determine whether there was any influence of the breed; we found that there were no significant differences in any of the parameters investigated among the groups. After puberty, the growth of the primordial follicles to the secondary follicle stage in the evaluated breeds would probably be similar, as confirmed by the similar number of secondary follicles recovered after follicular isolation. A similar follicular recovery suggests that the follicles are being formed in the same proportion in both breeds and that the *in vivo* dynamics of development of preantral follicles is possible similar to antral follicles in these populations (Cruz *et al*., 2005).

Regarding hormone production, the preantral follicles cultured *in vitro* in both groups tested showed steroid production and were able to secrete E2 in increasing levels between days 2 and 18. The major characteristics of the non-atretic growth and preovulatory ovarian follicles were the expression of P-450 aromatase and, thus, the ability to produce E2 (Bao *et al*., 1997). In addition, the continuation of E2 and P4 secretion suggested that the physiological function of the follicles, i.e., the interactions among the oocytes and surrounding follicular cells (granulosa cells and theca cells) were maintained (Adriaens *et al*., 2004). This finding suggests, for the first time, that the preantral follicles isolated from pure-breed (Saanen) or crossbreed (F1 generation: ½ Saanen + ½ Anglo-Nubian) showed similar hormone secretion patterns, which can be confirmed by the similar rates of follicular development and survival. In conclusion, under our experimental conditions, goat secondary follicles from Saanen or crossbreed (F1 generation) showed a similar pattern of *in vitro* follicular growth. This suggests that they can be used with the same efficiency as ovarian donors for *in vitro* culture of preantral follicles.

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