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Follicular interactions affect the *in vitro* development of isolated goat preantral follicles

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Summary

The aim of this study was to evaluate the influence of the number of follicles per drop (one or three) and antral follicles on *in vitro* development of isolated goat preantral follicles. Preantral follicles were isolated through microdissection and distributed individually (control) or in groups of three follicles (treatment) in microdroplets of α-MEM with or without 1000 ng/ml follicle stimulating hormone (FSH) for Experiments 1 and 2, respectively. Experiment 3 was divided into four treatments according to the presence of one or three preantral follicles, associated or not with antral follicles. After culture, oocytes were retrieved from morphologically normal follicles and submitted to *in vitro* maturation (IVM) and live/dead fluorescent labelling. Results of Experiment 1 (basic medium without FSH) showed that culture of preantral follicles in groups enhances viability, growth and antrum formation after 12 days. However, in the presence of FSH (Experiment 2), only the recovery rate of fully grown oocytes for IVM was significantly affected by grouping of follicles. In Experiment 3, in general, co-culture of preantral follicles with an early antral follicle had a detrimental effect on viability, antrum formation and production of oocytes for IVM. In conclusion, the performance of *in vitro* culture of goat preantral follicles is affected by the number of follicles per drop, the presence of an antral follicle and FSH.

Keywords: Caprine, Growth, Maturation, Oocyte, Viability

Introduction

Reproductive biotechniques used for *in vitro* embryo production depend on fertilizable oocytes, which are present in limited numbers in the ovary and are enclosed in preovulatory follicles. In this context, the development of an *in vitro* culture system capable of promoting the growth and development of preantral follicles for the retrieval of competent mature oocytes could enable the production of a vast number of embryos from a single ovary. This fact would cause a profound impact on fundamental research through a better comprehension of the processes comprising folliculogenesis and could also be practically applicable in clinics. This efficient system could be employed for the treatment of infertility in humans, avoiding intensive ovarian stimulations, as well as in domestic animals in order to maximize the reproductive potential of genetically valuable individuals (Demeestere *et al*., 2003; Haidari *et al*., 2008).

Since methods for isolation and *in vitro* culture of preantral follicles from mammalian ovaries were established, several culture systems have been proposed for the study of the complex mechanisms regulating follicular growth and oocyte maturation *in vitro*. The effects of some factors apparently essential for the success of *in vitro* development have been evaluated,

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such as the culture of follicles in group or individually (Spears *et al*., 1996; Wu *et al*., 2001; Gupta *et al*., 2002) and culture medium composition, which has been supplemented with antioxidants, growth factors and hormones (Eppig & O'Brien, 1996; O'Brien *et al*., 2003; Mao *et al*., 2004; Demeestere *et al*., 2005; Han *et al*., 2006; Gao *et al*., 2007; Gupta *et al*., 2008).

Some authors have demonstrated the importance of grouping preantral follicles retrieved from domestic animals for the *in vitro* culture, which increases growth rates in comparison with the culture of single follicles (Wu *et al*., 2001; Gupta *et al*., 2002). On the other hand, Spears *et al*. (1998) assessed the influence of interactions between follicles on *in vitro* growth until the antral stage, and demonstrated the occurrence of dominance in pairs of follicles cultured in contact. These authors hypothesized a specific interaction between adjacent follicles which determines the success of dominant follicle development.

Another important factor for the success of preantral follicles culture is the medium composition in which some key components, such as follicle stimulating hormone (FSH), play crucial roles. The *in vitro* development of preantral follicles is a complex process that comprises interactions of regulatory factors and hormonal signals. Ovarian function is critically regulated by FSH, which controls steroidogenesis, the proliferation and differentiation of granulosa cells, the development of antral follicles and the selection of dominant follicles (Baker & Spears, 1999; Demeestere *et al*., 2005). Due to its pivotal functions, FSH has been included in the culture of preantral follicles of mice and other mammals (Nayadu & Osborn 1992; Cortvrindt *et al*., 1996; Mao *et al*., 2002).

Few studies have been reported regarding the *in vitro* development of isolated caprine preantral follicles. Moreover, the factors required in the production of competent oocytes for fertilization and *in vitro* embryo development are not fully defined. In this context, the aim of this study was to evaluate the influence of number of follicles per drop (one or three) and antral follicles on *in vitro* development of isolated goat preantral follicles.

Materials and methods

Chemicals

Unless mentioned otherwise, culture media, supplements and chemicals used in the present study were purchased from Sigma Chemical Co.

Source of ovaries

Caprine ovaries ($n = 32$) were obtained at a local slaughterhouse from adult cross-breed goats ($n = 16$). Immediately postmortem, ovaries were washed once in 70% alcohol for 10 s and then twice in minimum essential medium (MEM) containing HEPES and antibiotics (100 μg/ml penicillin and 100 μg/ml streptomycin) and transported within 1 h to the laboratory in MEM at 4°C.

Culture medium

The culture medium was alpha-MEM supplemented with 1.25 mg/ml bovine serum albumin (BSA), ITS (10 μg/ml insulin, 5.5 μg/ml transferrin and 5 ng/ml selenium), 2 mM glutamine, 2 mM hypoxanthine and 50 μg/ml ascorbic acid. For Experiments 2 and 3, the same medium was supplemented with recombinant FSH (Nanocore, Brazil) at the concentration of 1000 ng/ml.

Isolation and selection of preantral follicles

At the laboratory, the ovaries were stripped of surrounding fat tissue and ligaments, and thin (1 mm) slices from the ovarian surface were made using a surgical blade and washed in fragmentation medium (MEM HEPES) with antibiotics. Preantral follicles larger than 150 μm in diameter with no antral cavities were visualized under a dissecting microscope and isolated using 26G needles attached to a syringe, and then transferred to the culture medium for quality evaluation. Isolated follicles from different ovaries were pooled and only preantral follicles with a centrally located oocyte surrounded by various compact layers of granulosa cells and no damage in the basal membrane were selected for culture.

Experimental design

Experiment 1 was carried out to verify the effect of individual or group culturing of isolated follicles in medium without FSH. Selected preantral follicles were randomly distributed in 40 μl microdroplets of cultured medium covered with mineral oil in culture dishes (60 × 15 mm) at 39°C in a humidified atmosphere of 5% CO_2 in air. Individually cultured follicles (one follicle per drop) represented the control, and groups of three follicles per drop were considered as the treatment group. The culture media were prepared daily and kept for equilibration in a CO_2 incubator for at least 2 h before using for culture. Every alternate day, medium was partially replaced (20 μl per drop). The day on which the follicles were placed in culture was taken as day 0 (D0) and subsequent days as day 2 (D2), day four (D4), and so on.

In Experiment 2, to determine the effect of individual (one follicle per drop) and group (three follicles per drop) culture of isolated follicles in medium with FSH, preantral follicles were cultured under the same conditions as described above except for the addition of FSH (1000 ng/ml) to the culture medium.

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In Experiment 3, to evaluate the effect of follicular dominance of early antral follicles over preantral follicles *in vitro*, the latter were cultured individually or in groups (three follicles per drop) in the presence or absence of an early antral follicle with a diameter of approximately 330 μm in medium containing 1000 ng/ml FSH. Early antral follicles and preantral follicles were dissected individually as described in Experiments 1 and 2. Isolated follicles were distributed in 40 μl microdroplets of the same medium used in Experiment 2. Treatment 1 consisted of one follicle per drop; treatment 2, one follicle per drop in co-culture with an early antral follicle; treatment 3 was carried out with three follicles per drop; treatment 4, group of three follicles co-cultured with an early antral follicle.

Morphological evaluation of follicle development

The viability of all of the preantral follicles was assessed every 6 days using a precalibrated ocular micrometer in a stereomicroscope (SMZ 645, Nikon) at ×20 magnification. Follicle degeneration was recognized through observation of rupture of the basement membrane, opacity of granulosa cells and/or extrusion of the oocyte from the follicle.

Follicle diameter and growth rate were recorded every 6 days by means of two perpendicular measures of each preantral follicle. Growth rate was calculated as the diameter variation during the culture period. The interval of 6 days of culture was chosen for diameter measurements because preliminary studies in our laboratory showed that no significant changes in follicle size occurred over a period shorter than 6 days. Antrum formation was considered as soon as small patches of antral cavities appeared.

Retrieval of oocytes from *in vitro* grown preantral follicles and viability assessment

At the end of culture, oocytes were dissected out from follicles with fine needles (26G) under a stereomicroscope from all of the surviving follicles. Previous studies demonstrated that caprine oocytes smaller than 100 μm were not able to resume meiosis (Crozet *et al*., 2000). Based on that finding, only oocytes larger than 110 μm were used for the *in vitro* maturation (IVM) procedure in our study. Oocytes with homogenous ooplasm and at least one layer of compact cumulus cells were considered acceptable for IVM. The recovery percentage of oocytes was calculated as the number of acceptable quality oocytes recovered out from the total number of cultured follicles. Collected oocytes were washed in IVM medium comprising TCM199 supplemented with 100 μg/ml luteinizing hormone (LH), 10% fetal bovine serum, 5 μg/ml FSH, 10 ng/ml epidermal growth factor, 22 μg/ml pyruvate and 1 μg/ml estradiol and placed in groups into 50 μl droplets of the same medium, previously equilibrated at 39°C under a humidified atmosphere of 5% CO_2 in air. These droplets were covered with mineral oil and the oocytes were incubated under the same conditions for 26 to 30 hours.

After IVM, oocytes were denuded and live/dead fluorescence labelling was performed in 100 μl droplets of TCM-HEPES containing 4 μM calcein-AM, 2 μM ethidium homodimer-1 (Molecular Probes, Invitrogen), and 10 μl of Hoechst 33342 stain followed by an incubation at 37°C for 15 min. Finally, oocytes were mounted on glass slides and examined using a DMLB fluorescence microscope (Leica). The emitted fluorescent signals of Hoechst stain, calcein-AM and ethidium homodimer were collected at 350, 488 and 568 nm, respectively. While the first probe detected intracellular esterase activity of viable cells, the latter labelled the nucleic acids of non-viable cells with plasma membrane disruption. Oocytes were considered live if the cytoplasm was stained positively with calcein-AM (green) and if chromatin was not labelled with ethidium homodimer (red).

Statistical analysis

The percentages of follicular survival, antrum formation and maturation rates were compared by chi-squared test (StatView for Windows). Follicular diameter and growth rate after culture were compared by ANOVA and Kruskal–Wallis test. Values were considered statistically significant when $p < 0.05$.

Results

Experiment 1: The effect of individually (one follicle per drop) and group (three follicles per drop) cultured isolated preantral follicles in medium without FSH

Viability

A total of 54 follicles were cultured for 22 days in Experiment 1. Immediately after isolation, preantral follicle granulosa cells surrounded a spherical oocyte, and the granulosa cells were limited by an intact basement membrane (Fig. 1). The follicular survival decreased significantly during the culture period (Fig. 2). There was no significant difference in the survival rate of the follicles when they were cultured in groups or alone in the microdrop until 12 days of culture. However, on D18, there were no viable follicles in the control group while 22.2% of the follicles cultured in group survived.

Follicle diameter and growth rate

The mean diameters of the preantral follicles at the onset of the culture (D0) were 178.76 ± 40.9 μm and 180.02 ± 36.6 μm in the control and test group, respectively, which were similar to each other ($p < 0.05$)

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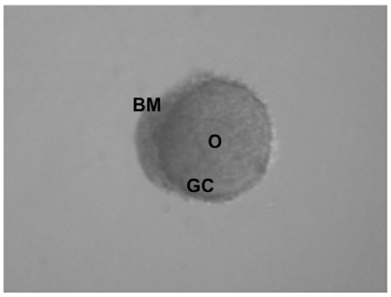

Figure 1 Isolated caprine preantral follicle on D0. BM, basement membrane; GC, granulosa cells; O, oocyte.

until D6 of culture. However, the diameter of follicles cultured in groups was significantly higher than that of the control from D12 of culture onwards (Fig. 3).

Growth rates of follicles cultured in groups were also significantly higher (21.4 ± 11.6 μm) than those of individually cultured follicles (13.6 ± 8.1 μm, $p < 0.05$).

Antral cavity formation

Antrum formation was detected through the visualization of translucent cavities using a stereomicroscope, and began on D2 of culture in follicles cultured in group and on D4 in the control (Fig. 4). A significantly higher percentage of antral cavity formation was observed in follicles cultured in groups (51.85%) in comparison with the control (22.2%) on D6 of culture. Nevertheless, from D12 of culture onwards, no significant difference was observed between the control (29%) and test group (55.6%, $p < 0.05$, Fig. 5).

Oocyte recovery after culture

In this experiment, oocytes did not reach the minimum diameter (110 μm) for IVM.

Experiment 2: The effect of individually (one follicle per drop) and group (three follicles per drop) cultured isolated preantral follicles in medium with FSH added

Viability

Percentages of viable follicles after individual (control) or group culture (treatment 2) were similar. In contrast to the results of Experiment 1 (culture without FSH), in which a significant decrease of the percentages of viable follicles was observed from D6 of culture onwards in both treatments, the use of FSH sustained viability until the same day of culture. Viability was significantly decreased in individually cultured follicles from D12 to D18 of culture, while percentages of viable follicles in groups were maintained from D12 until D24 of culture (Fig. 6).

Follicle diameter and growth rate

The mean diameters of follicles in both treatments were similar throughout the *in vitro* culture. Follicle growth rates in the control and test group were 19.29 ± 9.51 μm/day and 20.07 ± 9.52 μm/day, respectively, and did not differ statistically. With regard to the period of culture, follicle diameters increased significantly from D6 to D12 of culture in both treatments, but only follicles cultured in

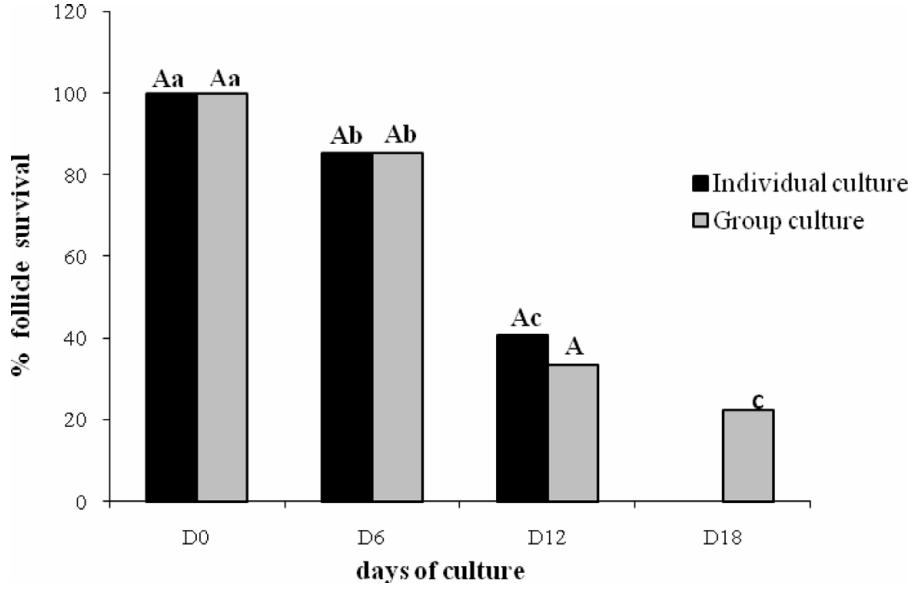

Figure 2 Percentages of follicular survival during 18 days of culture of preantral follicles cultured individually (control) or in groups (treatment) in medium without FSH (Experiment 1). A,B – indicates the significance between treatments on each day of culture; a,b,c – indicates the significance between the days of culture within each treatment.

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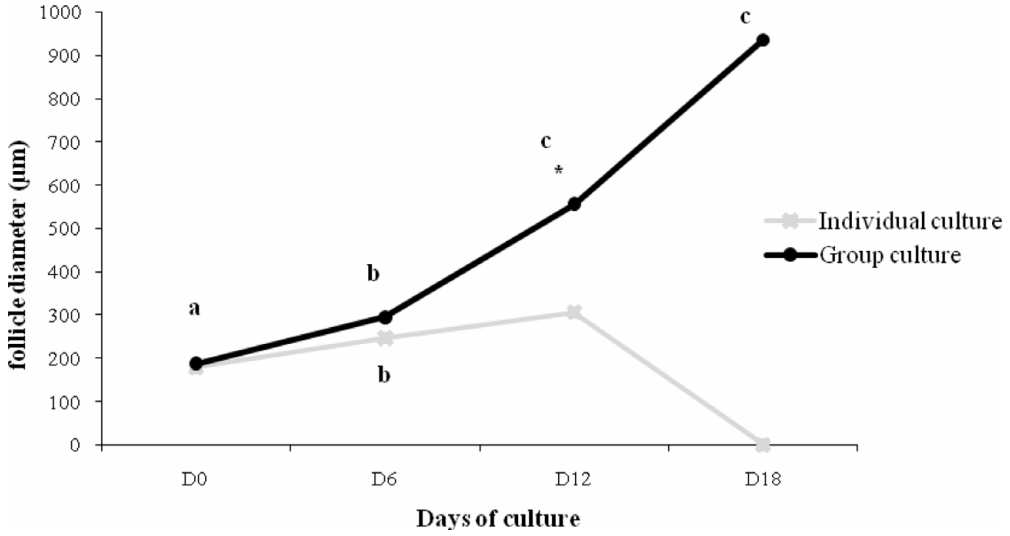

Figure 3 Diameter of follicles cultured individually (control) or in groups of three (treatment) in the absence of FSH (Experiment 1). * Indicates the significance between treatments from this day of culture onwards. a,b,c – indicates the significance between the days of culture within each treatment.

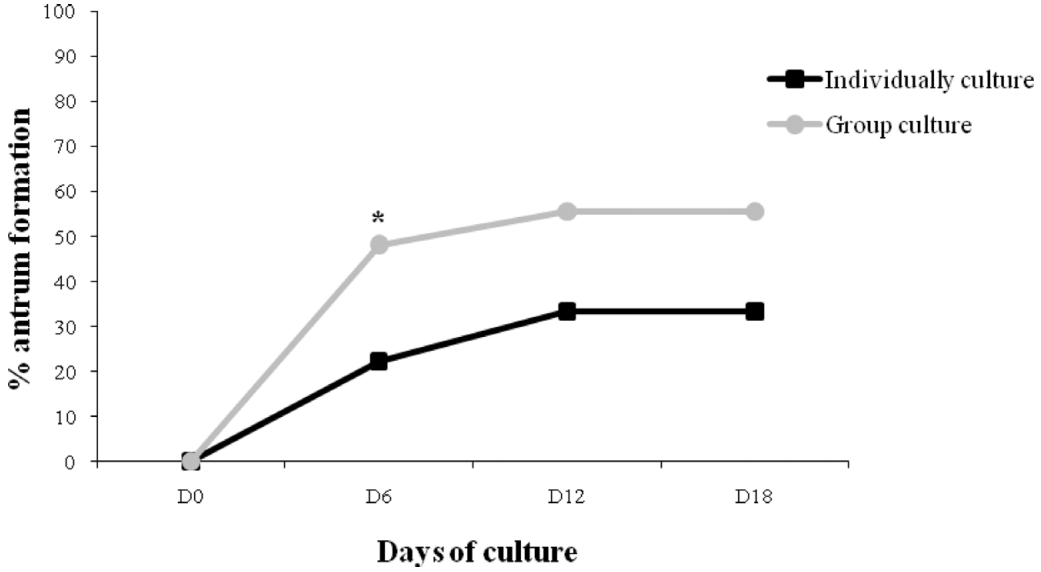

Figure 4 Antrum formation in follicles cultured individually (control) or in groups of three (treatment) in the absence of FSH (Experiment 1). *Indicates the significance between treatments on this day of culture.

groups showed a significant growth from D12 to D18 (Fig. 7).

Antral cavity formation

Percentages of follicles showing antrum formation were similar between the individual (control) and group culture ($p < 0.05$) during the entire experiment (Fig. 8).

Oocyte recovery after culture

After 24 days of culture, a total of 33 oocytes reached the minimum diameter for IVM (≥110 µm) in both treatments. In the group of follicles cultured individually ($n = 38$), oocytes were recovered from 11 follicles (28.95%), while group cultured follicles yielded a higher rate of oocyte recovery (62.86%, 22 oocytes, $p < 0.05$). After IVM, 9.09% of the oocytes retrieved from follicles cultured in groups resumed meiosis and reached the metaphase I and anaphase I stages (Fig. 9*A*–*C*).

Experiment 3: The effect of early antral follicle co-culture with individually (one follicle per drop) and group (three follicles per drop) cultured isolated preantral follicles in medium with FSH

Viability

Individual × antral. After 12 and 24 days of culture, a higher percentage of viable single preantral follicles was observed in the group cultured without antral

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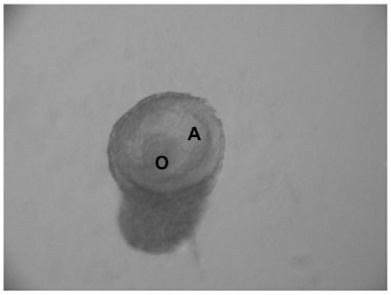

Figure 5 Antrum formation in a preantral follicle. A, antral cavity; O, oocyte.

follicles (control group) ($p < 0.05$). Viability was sustained in the control until D12 of culture, while co-culture with antral follicles significantly reduced the percentages of viable follicles from D6 to D12, and at the end of the culture (D24), viability in this group was only 23% (Fig. 10).

Group × antral. The presence of an antral follicle positively influenced the viability of preantral follicles cultured in groups, which was significantly higher (76%) on D18 of culture in comparison with the treatment without antral follicles (37.5%, $p < 0.05$). The latter group showed a significant reduction of viability from D12 to D18 of culture, while in the former treatment, percentages of viable follicles were decreased only on D24 (Fig. 11).

Follicle diameter and growth rate

Individual × antral. The diameters of follicles cultured individually were similar to those co-cultured with an antral follicle throughout the experiment, except on D12, when single follicles presented significantly higher diameters (Fig. 12, $p < 0.05$).

Group × antral. Although no significant difference in follicle diameter was observed between the treatments, a significantly higher growth rate was achieved in preantral follicles cultured without an antral follicle (control × group = 24.9 ± 9 μm/day) in comparison with the co-culture group (group × antral = 19.4 ± 6.6 μm/day) (Fig. 13).

Antrum formation

Individual × antral. The percentage of antrum formation was higher on D12 onwards in follicles cultured without an antral follicle compared with those co-cultured with an antral follicle ($p < 0.05$). During the culture period, the control group showed a significant increase in the percentage of antral cavity formation from D6 of culture, and this percentage became higher on D12. However, in the group co-cultured with the antral follicle, the significant increase was observed on D6 but was sustained until the end of culture (Fig. 14).

Group × antral. Antrum formation rate was not affected by the presence of an early antral follicle during *in vitro* culture of follicles in groups (Fig. 15).

Oocyte recovery after culture

Individual × antral. The culture of follicles individually allowed the retrieval of a significantly higher number of adequate oocytes for IVM (21 oocytes

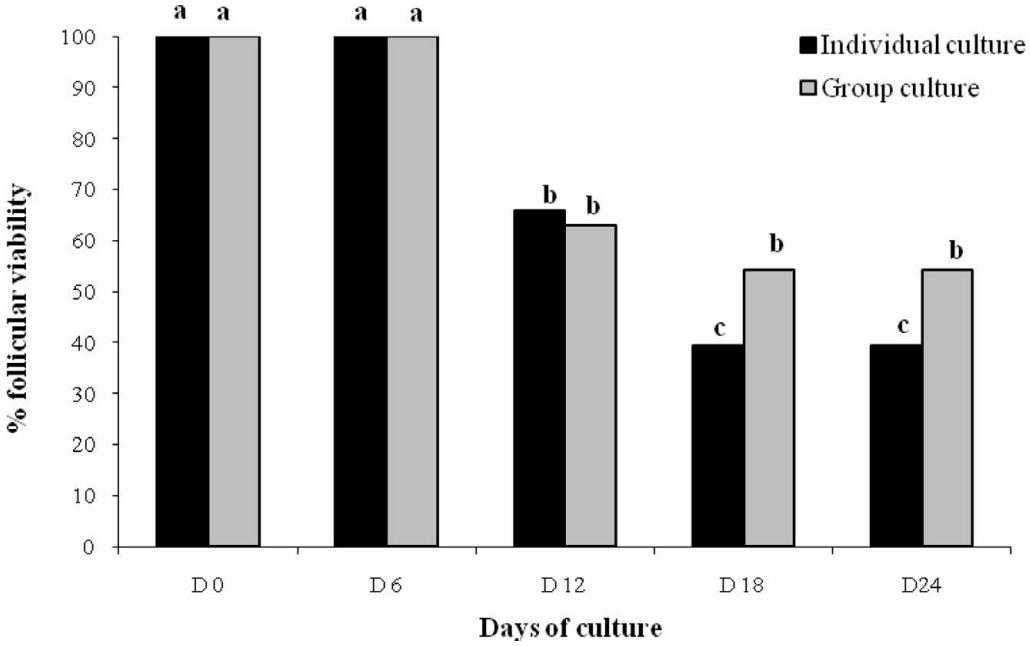

Figure 6 Percentages of follicular survival during the 18 days of culture of preantral follicles cultured individually (control) or in groups (treatment) in medium with FSH (Experiment 2). a,b,c – indicates the significance between the days of culture within each treatment.

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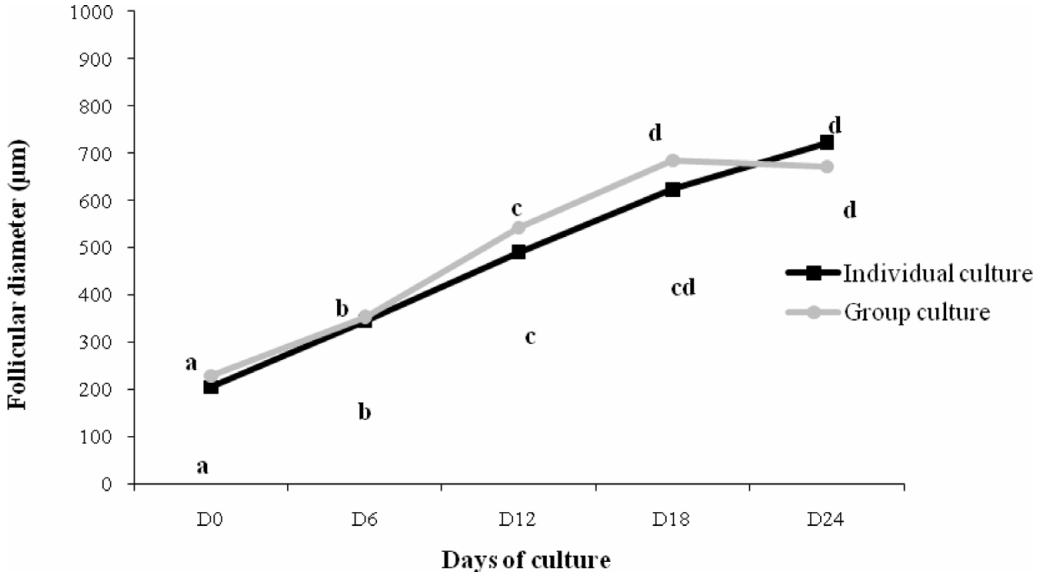

Figure 7 Follicular diameters of preantral follicles cultured individually (control) or in groups (treatment) in medium with FSH (Experiment 2) over 24 days. a,b,c,d – indicates the significance between the days of culture within each treatment.

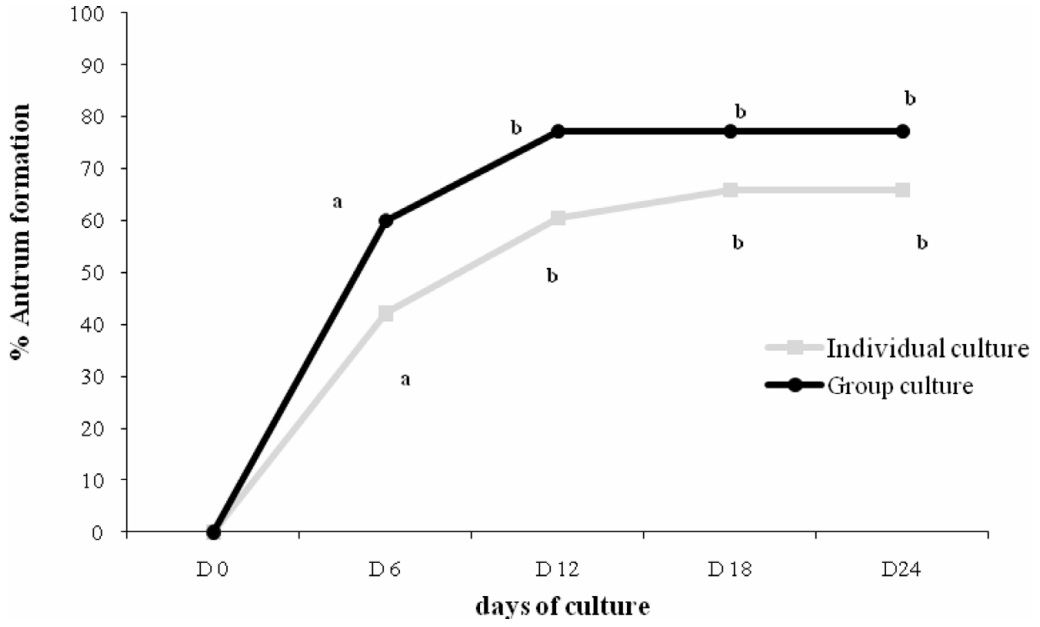

Figure 8 Antrum formation in follicles cultured individually (control) or in groups of three in medium with FSH (Experiment 2). a,b – indicates the significance between the days of culture within each treatment.

measuring at least 110 µm, 61.8% of the total of cultured preantral follicles) than co-culture with an early antral follicle (12 oocytes, 34.3% of the total of cultured preantral follicles, $p < 0.05$). After IVM, one oocyte recovered from follicles cultured individually (control) reached the metaphase II stage (Fig. 16*E*,*F*), while one oocyte from the treatment group that was co-cultured with an antral follicle resumed meiosis until the metaphase I stage (Fig. 16*A*,*B*).

Group × antral. With respect to the number of oocytes recovered for IVM, no statistical difference was found between follicles cultured in groups with or without an antral follicle (three oocytes in the control, 46.8%; three oocytes in the test group, 44.7%). Resumption of meiosis (metaphase I) was observed with oocytes in both treatment groups.

Discussion

The present study evaluated, for the first time, the effects of interactions between caprine preantral follicles on their *in vitro* growth, through the comparison of

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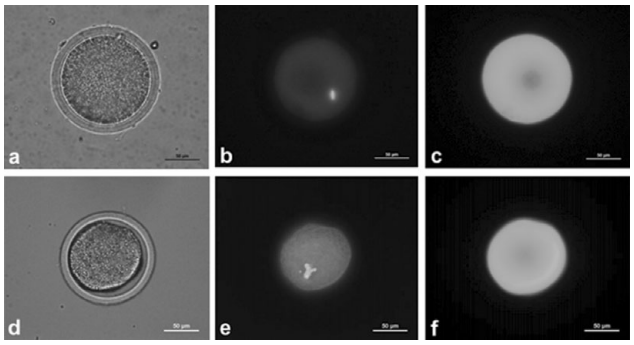

Figure 9 Meiosis resumption in oocytes recovered from preantral follicles cultured in groups in medium containing FSH (Experiment 2). A viable metaphase I oocyte (*a*–*c*) and a viable anaphase I oocyte (*d*–*f*) labelled with Hoechst stain (*b*, *e*) and calcein-AM (*c*, *f*). Scale bars represent 50 μm.

individual follicle and group culture. The influence of FSH and co-culture with early antral follicles on these interactions was assessed. The described model is a valuable tool to study the interactions and dominance between follicles *in vitro*.

The results of Experiment 1 showed that grouping caprine preantral follicles for *in vitro* culture without FSH enhances viability maintenance, growth and antrum formation after 12 days of culture. Several studies have demonstrated that culturing embryos in group increases cleavage and blastocyst rates, which may be due to the production of autocrine and paracrine factors that stimulate embryo development *in vitro* (Gardner & Lane, 1993; Pugh *et al*., 1993; Trounson *et al*., 1994; Fukui *et al*., 1996). Some works have also shown that grouping preantral follicles for *in vitro* culture results in higher growth rates, and an ideal number of follicles in each culture drop stimulates their development through a paracrine interfollicular regulation involving secretion of growth factors (Wu *et al*., 2001; Gupta *et al*., 2002). In addition to the importance of co-cultured follicles, the volume of medium may also influence paracrine regulation. Secreted factors which stimulate growth can be diluted if large medium amounts are used, so it is necessary to balance numbers of follicles and medium volume. Low volumes of medium for group culturing of mouse, ovine and bovine embryos are beneficial for the paracrine interactions that stimulate development (Fukui *et al*., 1996). Follicular culture in groups may also be detrimental as excessive numbers of follicles can lead aggregation in single masses which can inhibit growth and development. Wu *et al.* (2001) observed that the culture of five or more follicles per drop results in contact and subsequent growth suppression.

In Experiment 2, group culturing enhanced viability maintenance in the presence of FSH from day 18 onwards, while percentages of viable follicles cultured individually decreased significantly after culture for 12 days. The addition of FSH to the medium also to sustain viability in both treatments, since 39.5% and 54% viable follicles were observed after 18 days in the control and group culture treatment, respectively, while in Experiment 1 (culture without FSH), a viability of only 22% was observed in the follicles cultured in groups, and not a single viable follicle could be found in the control at the same culture period. These results are in accordance with other

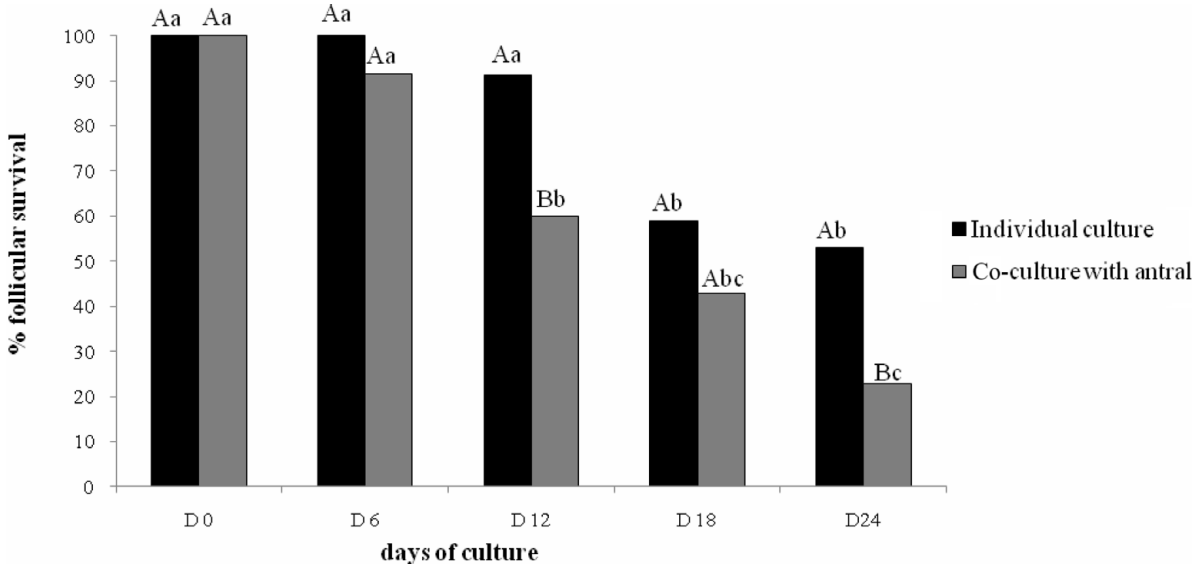

Figure 10 Percentages of follicular survival during the 24 days of culture of preantral follicles cultured individually (control) or with antral follicles (treatment) (Experiment 3). A,B – indicates the significance between treatments on each day of culture; a,b,c – indicates the significance between the days of culture within each treatment.

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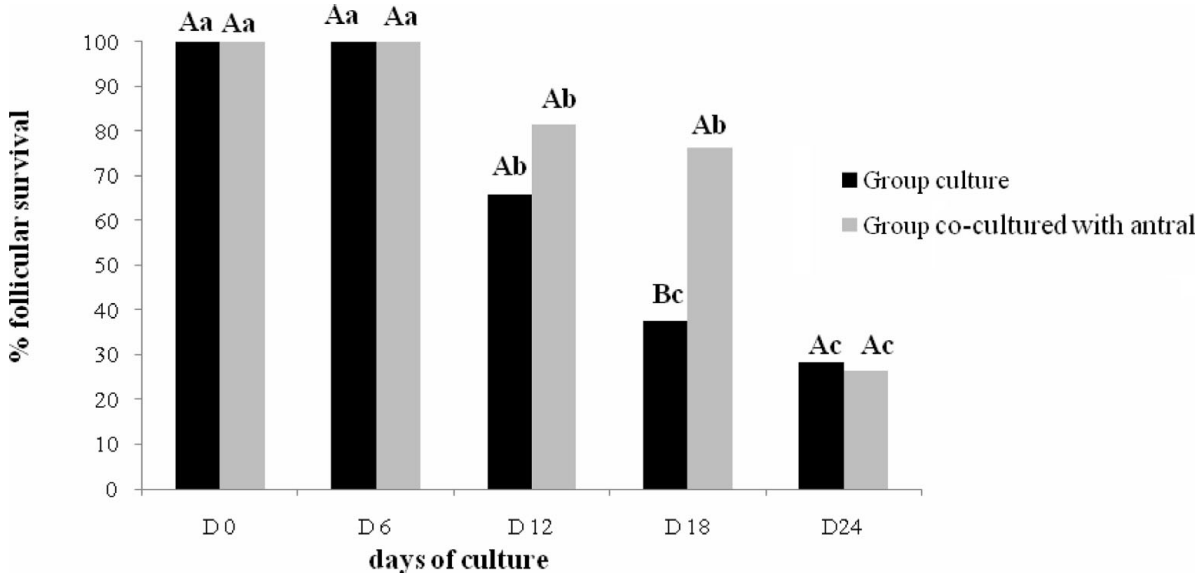

Figure 11 Percentages of follicular survival during the 24 days of culture of preantral follicles cultured in groups (control) or in groups co-cultured with antral follicles (treatment) (Experiment 3). A,B – indicates the significance between treatments on each day of culture; a,b,c – indicates the significance between the days of culture within each treatment.

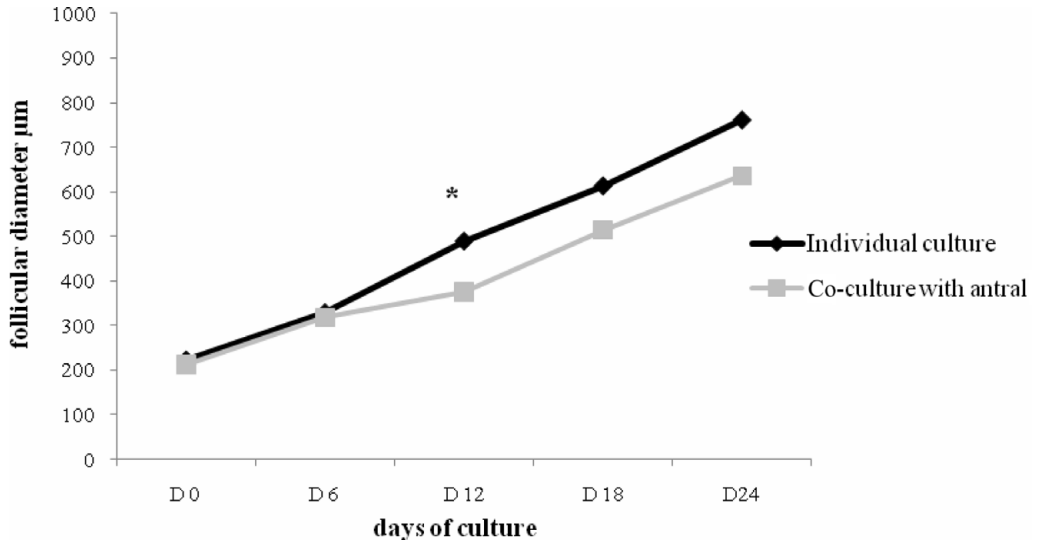

Figure 12 Follicle diameters during the 24 days of culture of preantral follicles cultured individually without (control) or with antral follicles (treatment) (Experiment 3). *Indicates the significance between treatments on this day of culture.

studies, in which only 10–17% of murine preantral follicles survived after *in vitro* culture without FSH, and a complete differentiation occurred only when this hormone was added to the culture during the advanced preantral stage (Cortvrindt *et al*., 1997; Mitchell *et al*., 2002; Adriaens *et al*., 2004).

The role of FSH in folliculogenesis is well known. *In vivo*, this hormone is essential for steroidogenesis through the stimulation of the enzyme aromatase, differentiation of granulosa cells and the induction of the expression of LH receptors. Regulation of intercellular junctions between the oocytes and surrounding granulosa cells is also performed by FSH (Albertini *et al*., 2001). Moreover, gonadotrophins activate the expression of anti-apoptotic proteins in granulosa cells *in vivo* and *in vitro* (Wang *et al*., 2003).

Under action of FSH (Experiment 2), group culture significantly increased follicle diameter until day 18 of culture. Several authors have reported that co-culture of isolated follicles is beneficial for *in vitro* growth (Gutierrez *et al*., 2000; Gupta *et al*., 2002). Wu and *et al*. (2001) demonstrated that culturing porcine preantral follicles in groups of three follicles promoted the highest growth rates in comparison with the incubation of one or five follicles, which suggests an ideal number of follicles in a culture

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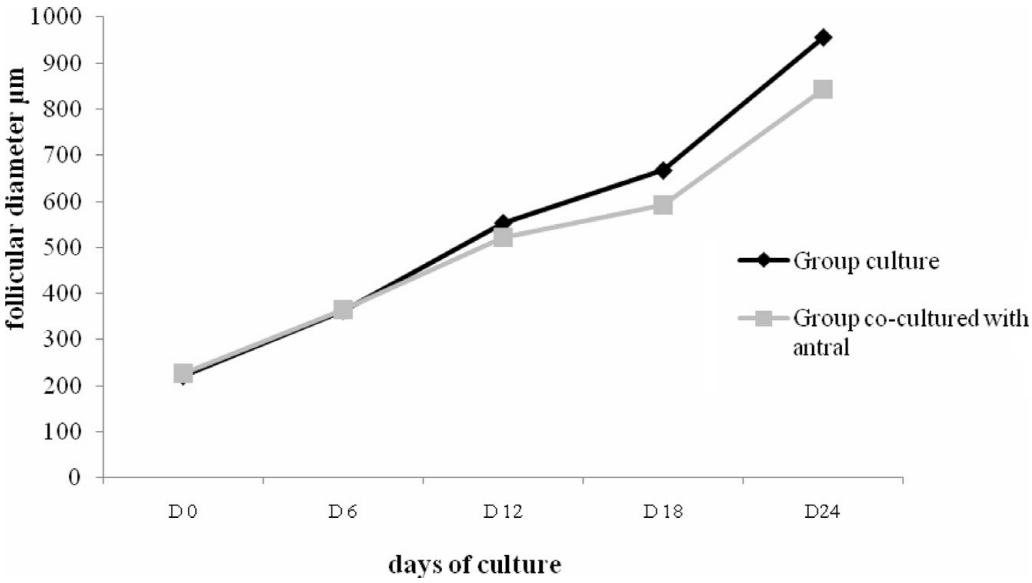

Figure 13 Follicle diameter during the 24 days of culture of preantral follicles cultured in groups without (control) or with antral follicles (treatment) (Experiment 3).

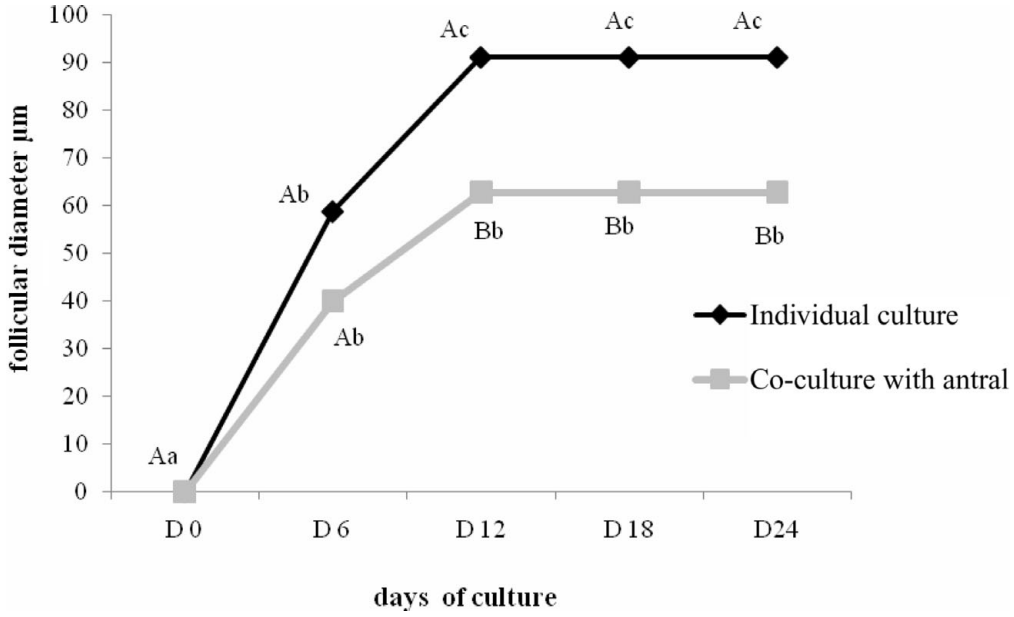

Figure 14 Antrum formation during the 24 days of culture of preantral follicles cultured individually without (control) or with antral follicles (treatment) (Experiment 3). A,B indicates the significance between treatments on each day of culture. a,b,c indicates the significance between the days of culture within each treatment.

drop for an optimum development by means of paracrine regulation. Spears *et al*. (1996) reported that dominant follicles from mice can suppress the growth of contacting follicles, but group culturing these follicles without contact is favourable for *in vitro* growth. Zhou & Zhang (2006) cultured caprine follicles in microdrops and observed the aggregation of follicles which could grow within the resulting masses. This condition proved to be beneficial for oocyte growth probably because a close contact among granulosa cells is achieved, which may be essential for the growth of oocytes *in vitro*. Such tight association might assist gap junctions in the transport of small molecules from granulosa cells to the oocytes (Eppig, 1991).

The competence to resume meiosis is progressively acquired in caprine oocytes during the late phase of follicular growth, together with the capability to be fertilized and to sustain early embryo development. Only a small proportion of oocytes retrieved from caprine antral follicles is able to support *in vitro* embryo development (Crozet *et al.*, 1995). In the present study, culture of preantral follicles in groups

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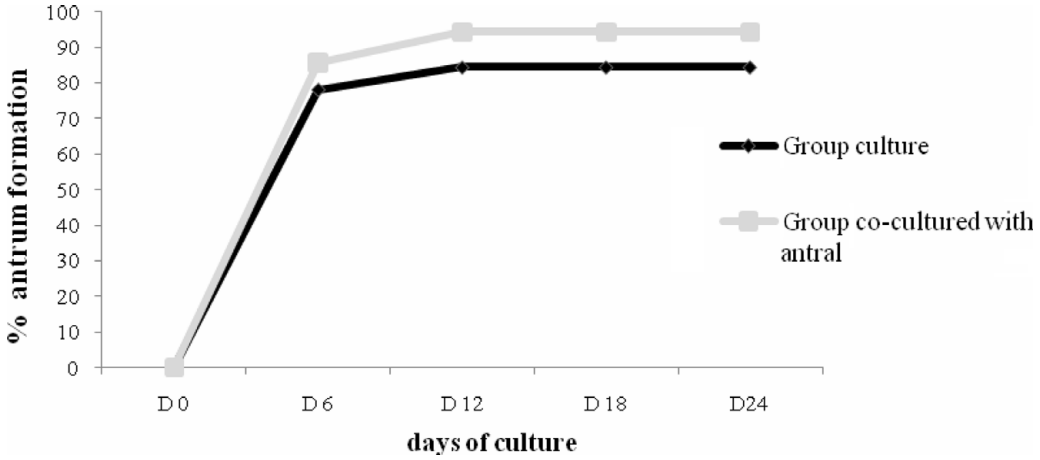

Figure 15 Antrum formation during the 24 days of culture of preantral follicles cultured in groups without (control) or with antral follicles (treatment) (Experiment 3).

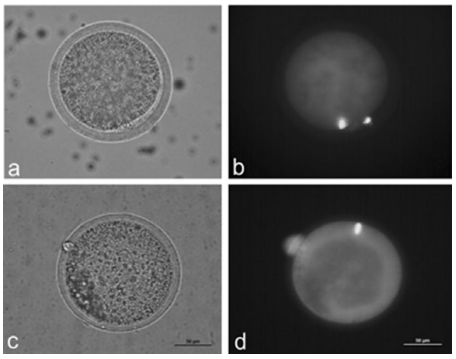

Figure 16 Meiosis resumption of oocytes recovered from preantral follicles cultured individually or in presence of an antral follicle in medium containing follicle stimulating hormone (FSH) (Experiment 3). MII oocyte from preantral follicle cultured for 24 days individually (*a*); and labelled with Hoechst stain (*b*); MI oocyte from preantral follicle after 24 days of in group culture in presence of an antral follicle (*c*); and labelled with Hoechst stain (*d*). Scale bars represent 50 μm.

positively influenced the *in vitro* growth of oocytes with the use of FSH (Experiment 2), making it possible to obtain oocytes with an adequate diameter (>110 μm) for IVM, which were able to resume meiosis. In this context, the development of an *in vitro* culture system capable of sustaining viability and promoting the growth of preantral follicles for longer periods will enable the retrieval of larger numbers of competent oocytes for the *in vitro* production of caprine embryos. Despite the fact that oocytes recovered in Experiment 1 were larger than 110 μm, a complete maturation (metaphase II) was not accomplished after IVM. Similarly to their counterparts in mice, cows and sows, caprine oocytes also need to acquire meiotic competence during the final phase of growth. Thus, a culture system capable of promoting higher growth rates for longer periods is required for adequate development and differentiation of oocytes, which need to express mRNA and synthesize specific proteins at a similar level as oocytes grown *in vivo* (De Smedt *et al.*, 1994; Crozet *et al.*, 2000).

In Experiment 3, co-culture of single preantral follicles with an early antral follicle had a detrimental effect on viability, growth, antrum formation and the production of oocytes with the minimum diameter for IVM. Nevertheless, preantral follicles cultured in groups benefited from the addition of an early antral follicle and presented higher viability, but no effect on antral cavity formation and retrieval of oocytes for IVM was observed. Conversely, the growth of these follicles was impaired, and significantly smaller diameters were found compared with groups of preantral follicles cultured without antral follicles.

Barker *et al.* (2001) observed that early antral follicles negatively influence the growth of mouse preantral follicles during *in vitro* culture, which demonstrates that dominance also occurs *in vitro* when follicles are co-cultured with others at different stages, and that larger follicles become dominant at the beginning of the culture and inhibit the growth of the others.

The process of follicle selection and dominance comprises complex interactions between a series of factors which are still not fully deciphered. The establishment of dominance and its maintenance are distinct events which can be studied separately. Interfollicular communication mediated through contact may act in the selection process by an inhibition of the development exerted over smaller follicles, and possibly the induction of a higher sensitivity to oscillations in FSH concentration (Baker & Spears, 1999; Baker *et al.*, 2001). Follicular dominance is controlled by a number of mechanisms acting together,

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including the production of local factors which can inhibit the growth of subordinate follicles without affecting viability. These inhibitory factors can act by systemically suppressing follicular growth in the contralateral ovary and can also exert local effects on the proliferation of granulosa cells and aromatase activity stimulated by FSH (Armstrong & Webb, 1997).

The development of subordinate follicles is suppressed through an endocrine mechanism in which inhibin and estradiol are secreted in increasing concentrations and downregulate the hypothalamus–hypophysis system. This downregulation leads to a decrease of circulating FSH, which in turn results in atresia of non-dominant follicles. In addition to this pathway, an intra-ovarian regulation of follicular dominance based on local inhibitory factors produced by follicles also has an important role. Such follicular interactions were evidenced by the results of the present study, in which the co-culture of preantral follicles in the same stage and an early antral follicle affected the *in vitro* development of preantral follicles.

Previous studies have shown that follicles can secrete factors which inhibit several aspects of the development of other follicles. Some works demonstrated a negative influence of antral cavity fluid on follicular development (Cahill *et al*., 1985). More recently, it was shown that low concentrations of follicular fluid from dominant follicles may contain beneficial factors for the growth of porcine preantral follicles, while fluid from smaller follicles can include compounds which inhibit the development of cultured preantral follicles (Metoki *et al*., 2008).

The findings of the present study (Experiment 3) indicate the existence of inhibitory factors produced by antral follicles which significantly slow down the growth of preantral follicles but do not induce atresia. In addition, this influence depends on the number of co-cultured follicles, since a negative effect on viability was observed only when a single preantral follicle was incubated with an antral follicle. Moreover, we hypothesize that a positive paracrine influence established among co-cultured preantral follicles counteracted the deleterious effect of the dominant follicle on viability observed in single preantral follicles co-incubated with an antral follicle.

Some elements, such as FSH concentration, can induce follicles to secrete stimulatory factors for other follicles in culture at the same developmental stage. Furthermore, FSH can suppress the inhibitory effect of factors produced by dominant follicles over *in vitro* co-cultured follicles in groups. During normal ovulatory cycles, the number of developing follicles can possibly be determined by a balance of local inhibitory and stimulatory factors and circulating levels of FSH at a given moment (Spears *et al*., 2002). Therefore, the presence of FSH or its concentration in the *in vitro* culture of follicles at different stages might influence such follicular interactions.

In conclusion, *in vitro* culture of caprine preantral follicles in groups enhances development in comparison with incubation of single follicles, and increases the production of competent oocytes for IVM. Furthermore, follicles at more advanced developmental stages can affect the *in vitro* growth of preantral follicles through interactions which depend on the number of co-cultured follicles. Future investigations are required to characterize the factors which determine dominance and other follicular interactions.

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