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In vitro development of secondary follicles from pre-pubertal and adult goats cultured in two-dimensional or three-dimensional systems

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Summary

The aim of this study was to evaluate the influence of two-dimensional (2D) and three-dimensional (3D) alginate culture systems on *in vitro* development of pre-antral caprine follicles. In addition, the influence of the reproductive age of the ovary donor on the *in vitro* culture success was investigated. Pre-antral follicles from pre-pubertal or adult goats were isolated and cultured directly on a plastic surface (2D) or encapsulated in an alginate-based matrix (3D). After 18 days, the oocytes underwent *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) to produce embryos. The 3D system showed higher rates of follicle survival, lower rates of oocyte extrusion, and a greater number of recovered oocytes for IVM and IVF ($P < 0.05$). Only pre-antral follicles from adult animals produced MII oocytes and embryos. The estradiol concentrations increased from day 2 to day 12 of culture in all groups tested ($P < 0.05$). Conversely, progesterone concentrations were lower in 3D-cultured follicles than in 2D-cultured follicles, with differences on days 2 and 6 of culture ($P < 0.05$). We provide compelling evidence that a 2D or 3D alginate *in vitro* culture system offers a promising approach to achieving full *in vitro* development of caprine pre-antral follicles to produce mature oocytes that are capable of fertilization and viable embryos.

Keywords: Alginate, Follicle culture, Oocyte, Pre-antral follicle, Steroid secretion

Introduction

In developing countries, such as Brazil, the goat industry is in considerable expansion and the use of assisted reproductive technologies may be an important way to increase the productivity of livestock in genetic improvement programmes (Guerra *et al*., 2011). *In vitro* culture of ovarian follicles has emerged as a potential reproductive technology for the production of large numbers of mature oocytes that are capable of fertilization (Demeestere *et al.*, 2005). In addition, the pre-antral follicle culture system has been suggested as a valuable *in vitro* assay to assess the influences of environmental mutagens, pharmaceutical agents and, potentially, endocrine-disrupting chemicals on follicular endocrine function and oocyte meiosis (Sun

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et al., 2004). In this regard, in order to evaluate follicle function and oocyte viability, data from hormonal production and fluorescence microscopy have been assessed in many studies in order to determine follicle health (Matos *et al.*, 2007). Although this biotechnology holds great promise, the successes reported have been limited to antrum formation in cattle (McLaughlin *et al.*, 2010) and bitches (Serafim *et al.*, 2010), oocyte maturation in primates (Xu *et al.*, 2011) and relatively low embryo production in buffaloes (Gupta *et al.*, 2008), sheep (Arunakumari *et al.*, 2010) and goats (Magalhães *et al.*, 2011).

Many factors influence the success of *in vitro* follicle development, including ovarian origin, culture medium components and the type of culture system used. Recently, using a two-dimensional (2D) culture system it was demonstrated that the addition of vascular endothelial growth factor (VEGF) to the culture medium improved oocyte meiotic resumption in secondary caprine follicles grown *in vitro* (Araújo *et al.*, 2011). In addition, this system was the first one to produce a caprine embryo after *in vitro* fertilization of oocytes derived from pre-antral follicles grown *in vitro* (Saraiva *et al.*, 2010). However, in 2D 'attachment' systems, only part of the follicle surface makes contact with the culture substrate on a flat tissue culture plate. As granulosa cells attach, the follicle flattens and expands outward. To maintain the spherical structure of the follicle, three-dimensional (3D) culture systems have been developed in which the follicle 'floats' in rotating tubes or inverted microdrops, or is encapsulated in a culture matrix, such as alginate (Brito *et al.*, 2013). Culture systems that utilize a 3D matrix to support follicles also increase the production of mature oocytes *in vitro*. Sodium alginate hydrogel matrices are able to maintain follicle structure and have been shown to support long-term cultures of primordial follicles (Hornick *et al.*, 2012). In mice, *in vitro* culture of pre-antral follicles in 3D alginate has resulted in mature oocytes that could be fertilized *in vitro* to produce viable offspring after embryo transfer (Xu *et al.*, 2006b). In monkeys, this system has supported the development of pre-antral follicles to the antral phase, with follicular hormone production similar to that seen *in vivo* (Xu *et al.*, 2009) and the production of fertilizable oocytes (Xu *et al.*, 2011). Despite these promising results, it is not known whether a 3D alginate culture system can improve the *in vitro* culture of isolated goat pre-antral follicles. Therefore, the aim of this study was to investigate the influence of 2D and 3D alginate culture systems on *in vitro* follicular development, viability, hormone production and the developmental competence of oocytes (embryogenesis) obtained from cultured pre-antral follicles. Moreover, the effect of the reproductive age of the ovary donor on the *in vitro* culture goat pre-antral follicles was also investigated.

Materials and methods

Animals and ovary collection

Ovaries ($n = 26$) from six pre-pubertal (5-month-old) and seven adult (1–5-year-old), cyclic, mixed breed goats (*Capra hircus*) that were controlled animals with a good body condition score and known age were collected from a local slaughterhouse. Immediately postmortem, each pair of ovaries were washed once in 70% alcohol and then twice in minimum essential medium (MEM) buffered with HEPES plus antibiotics (100 μg/ml penicillin and 100 μg/ml streptomycin) designed as MEM–HEPES and transported to the laboratory in wash medium at 4°C within 1 h of collection. Three batches of ovaries collections were processed during 3 days sequentially (four, four and five animals on the first, second and third days). Unless mentioned otherwise, supplements, hormones, and chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Isolation and selection of pre-antral follicles

In the laboratory, surrounding fat tissue and ligaments were stripped from the pre-pubertal and adult ovaries. Ovarian cortical slices (1–2 mm thick) were cut from the ovarian surface using a surgical blade under sterile conditions and subsequently placed in MEM–HEPES. Pre-antral follicles 150–250 μm in diameter without antral cavities (secondary follicles) were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) and mechanically isolated by microdissection from strips of ovarian cortex using 26-G needles, then transferred to 100-μl drops of α-MEM (Gibco, Invitrogen, Karlsruhe, Germany, pH 7.2–7.4) for quality evaluation. Only isolated secondary follicles that displayed the following characteristics: (i) an intact basal membrane; (ii) two to three layers of granulosa cells; and (iii) a visible, healthy oocyte that was round and centrally located within the follicle, without dark cytoplasm; were selected for culture.

Alginate hydrogel preparation and follicle encapsulation

We used an alginate matrix 3D culture system. Sodium alginate (55–65% guluronic acid) was provided by the Institute for Women's Health Research (Chicago, IL, USA). Alginate aliquots were reconstituted by mixing on a racking platform at room temperature overnight

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with sterile phosphate-buffered saline (PBS; 137 mM NaCl, 10 mM phosphate, and 2.7 mM KCl; Invitrogen) to a concentration of 0.5% (w/v).

After isolation and selection the follicles were encapsulated individually in 0.5% alginate beads. In detail, for alginate encapsulation, the follicles were washed in three drops (100 μl each) of alginate solution in Petri dishes (60 × 15 mm; Corning, USA). Then, a 5 μl alginate droplet that contained a single follicle was immersed in a cross-linking solution (50 mM $CaCl_2$ and 140 mM NaCl). Following cross-linking for 2 min, the alginate beads were removed and rinsed in α-MEM medium. Finally, each bead was transferred to a single well of a 48-well plate in 300 μl culture medium as described by Xu *et al.* (2009).

Culture of isolated goat pre-antral follicles

Secondary follicles from pre-pubertal (PP) and adult (AD) goat ovaries isolated, as described above, were cultured individually (1 follicle per drop) in a plastic (2D) or alginate (3D) culture system. All secondary follicles (n = 172) were from three replicates of the culture and the follicles were distributed in the following treatments: PP/2D ($n = 34$), PP/3D ($n = 49$), AD/2D ($n = 37$), AD/3D ($n = 52$).

The culture medium of both systems, hereafter referred to as α-MEM$^+$, consisted of α-MEM (Gibco, Invitrogen, Karlsruhe, Germany; pH 7.2–7.4) 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, 100 ng/ml VEGF and bovine recombinant follicle-stimulating hormone (FSH; Nanocore, São Paulo, Brazil) in increasing concentrations (day 0: 100 ng/ml; day 6: 500 ng/ml; day 12: 1000 ng/ml). The concentrations of VEGF and FSH were chosen based on previous studies performed in our laboratory (Araújo *et al.*, 2011). For the 2D culture system, the follicles were cultured in 100 μl drops of medium under mineral oil in Petri dishes (60 × 15 mm; Corning, USA). Every other day, 60 μl of the culture medium in each drop was replaced with fresh medium (prepared and kept for equilibration in a CO_2 incubator for 4 h prior to use). In the 3D alginate system, the follicles were cultured in 300 μl of culture medium in 48-well culture dishes (Corning, USA) and 150 μl of medium was exchanged every other day. The volume of medium replaced in the 2D (Araújo *et al.*, 2011) and in 3D systems (Xu *et al.*, 2009) was determined previously. Follicles were cultured at 39°C and in 5% CO_2 in air for 18 days. Every 2 days, medium collected was stored (volume of each sample: 60 μl) at –20°C for subsequent hormonal measurements.

Morphological evaluation of follicle development

Pre-antral follicle morphology was assessed every 6 days of culture and the follicular diameter was measured using a pre-calibrated ocular micrometer in a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) at ×100 magnification. Only those follicles that showed an intact basal membrane, bright and homogeneous granulosa cells, no dark oocyte and surrounding cumulus cells were measured. Follicles were classified as degenerated if the oocytes and surrounding cumulus cells appeared dark, oocytes were misshapen, or if the diameter of the follicle decreased.

The following characteristics of the surviving follicles were analysed every 6 days: (i) antral cavity formation, defined as the emergence of a visible translucent cavity within the granulosa cell layers; (ii) follicle extrusion, or the release of oocytes from the follicles; and (iii) follicle growth, two perpendicular diameters were recorded for each healthy follicle and the average of these two values was reported as the follicular diameter. The growth rate was calculated based on the change in follicle diameter during the 18-day culture period. The assessment interval of 6 days was chosen based on preliminary studies in our laboratory that showed no significant changes in follicle size during periods shorter than 6 days.

In vitro maturation (IVM), *in vitro* fertilization (IVF), and embryo production from the cultured pre-antral follicles

At the end of the 18-day culture period, cumulus–oocyte complexes (COCs) from all the healthy follicles were recovered by mechanically opening the follicles with a 26-G needle under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan). Previous studies have demonstrated that goat oocytes smaller than 110 μm were unable to resume meiosis (Crozet *et al.*, 2000), thus only oocytes larger than 110 μm in diameter (excluding the zona pellucida; ZP) with homogeneous cytoplasm and surrounded by at least one compact layer of cumulus cells were selected for IVM. The recovery rate of oocytes was calculated as the number of acceptable quality oocytes recovered (from follicles >110 μm) out of the total number of cultured follicles.

Collected COCs were washed twice with TCM199 medium buffered with HEPES (TCM199–HEPES) and placed into 50-μl droplets of IVM medium: TCM199 medium was supplemented with 10% fetal calf serum (FCS), 100 μg/ml luteinizing hormone (LH), 5 μg/ml FSH, 10 ng/ml epidermal growth factor (EGF), and 1 μg/ml 17β-estradiol. Prior to placement of COCs, the IVM medium was equilibrated at 39°C under a humidified atmosphere of 5% CO_2 in air. The individual COC-containing droplets were placed on

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culture dishes (30 × 15 mm), covered with mineral oil, and incubated for 32 h.

After IVM, oocytes were fertilized in base synthetic oviduct fluid medium with added amino acids (using SOFaa medium), 2% estrus goat serum, and 10 μg/ml heparin. The final concentration of sperm was 200 × 10^6 spermatozoa (Spz)/ml. Presumptive zygotes were cultured for 5 days in SOFaa medium with 10% fetal bovine serum (FBS); cleavage and embryo production were determined on days 2 and 5, respectively.

Assessment of oocyte viability and chromatin configuration

After the second embryo culture day, non-cleaved oocytes were transferred to 50 μl drops of TCM199 medium with 10 μl hyaluronidase (0.1%), and cumulus cells were removed by successive pipetting. The denuded oocytes were analysed for oocyte viability and chromatin configuration as follows: oocytes were incubated in 100 μl droplets of TCM199–HEPES that contained 4 μM calcein-AM, 2 μM ethidium homodimer-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany), and 10 μM Hoechst 33342 stain (Sigma, Deisenhofen, Germany) at 39°C for 15 min. The oocytes were then washed three times in TCM199, placed on glass slides, and examined under a fluorescence microscope (Nikon, Eclipse 80i, Tokyo, Japan). Calcein-AM detects intracellular esterase activity in viable cells, whereas ethidium homodimer-1 labels nucleic acids of non-viable cells with plasma membrane disruption. Oocytes were considered viable if the cytoplasm was positive for calcein-AM (green) and if the chromatin was not labelled with ethidium homodimer-1 (red). Hoechst staining was used to evaluate nuclear chromatin configuration, with the presence of either an intact germinal vesicle (GV), meiotic resumption (including germinal vesicle breakdown (GVBD) and metaphase I (MI)), or nuclear maturation (metaphase II (MII)).

Hormone assays

The hormonal analysis were performed using medium (60 μl each sample) collected on days 2, 6, 12 and 18 of culture and stored at –20 °C. Estradiol (E2) and progesterone (P4) secretion were determined by double antibody radioimmunoassay (RIA) using MP kits (MP Biomedicals, Solon, OH, USA). The volume of medium used for analyses was 60 μl. The lower detection limit and the intra-assay coefficient of variation were, respectively, 5 pg/ml and 2.5% for E2 and 0.01 ng/ml and 3.7% for P4. In order to prevent interassay variation, all samples were assayed in the same RIA.

Statistical analysis

Because follicle isolation procedures yield a different number of structures, follicles were pooled and considered to be the experimental unit, instead of the number of animals. Discrete valuables obtained by counting, such as follicular viability, antrum formation, oocyte extrusion, recovery rate, GV, and meiotic resumption were analysed by dispersion of frequency using the chi-squared test, and the results were expressed as percentages. Continuous variables, such as follicular and oocyte diameter, were analysed initially using Shapiro–Wilk and Bartlett's tests to verify the requirements underlying analysis of variance, i.e. normal distribution and homogeneity of variance. Homoscedasticity was not present, even after data transformation, so both follicular and oocyte diameters were assessed using the Kruskal–Wallis test. The results were expressed as mean ± standard error of the mean (SEM) and differences were considered to be significant when P-values were < 0.05.

Results

Follicular morphology, diameter and growth rate

Goat ovarian follicles cultured on 2D culture (Fig. 1*a–c*) or in 3D alginate (Fig. 1*d–f*), on day 0, day 12, and day 18 of culture are shown (Fig. 1). After 12 days on 2D culture, we observed follicle adherence (PP/2D = six follicles; AD/2D = nine follicles) to the culture plate that resulted in a loss of normal morphology by day 18, culminating in follicular degeneration (Fig. 1*c*). In contrast, the 3D alginate system maintained follicular morphology throughout the *in vitro* culture period (Fig. 1*f*).

For all treatments, follicular diameter increased significantly from day 0 to day 12 of culture (Table 1). However, the increase was only observed in PP and AD groups on 2D culture ($P < 0.05$) from day 12 to day 18. In fact, starting at day 12, a higher follicular diameter was observed in the 2D culture system compared with the 3D alginate system. Likewise, the follicle growth rates (μm/day) on 2D culture of pre-pubertal (17.77 ± 6.96) and adult (19.78 ± 9.76) follicles were higher ($P < 0.05$) than those of the 3D alginate-cultured groups (pre-pubertal: 11.54 ± 5.99; adult: 10.91 ± 5.91). The total number of follicles used per treatment was: PP/2D (n = 34), PP/3D (n = 49), AD/2D (n = 37), AD/3D (n = 52).

Survival of goat pre-antral follicles cultured *in vitro*

Survival ratio (%), oocyte ratio extrusion (%) and follicular diameter after *in vitro* culture are shown (Table 2). Compared with day 0, there was a reduction

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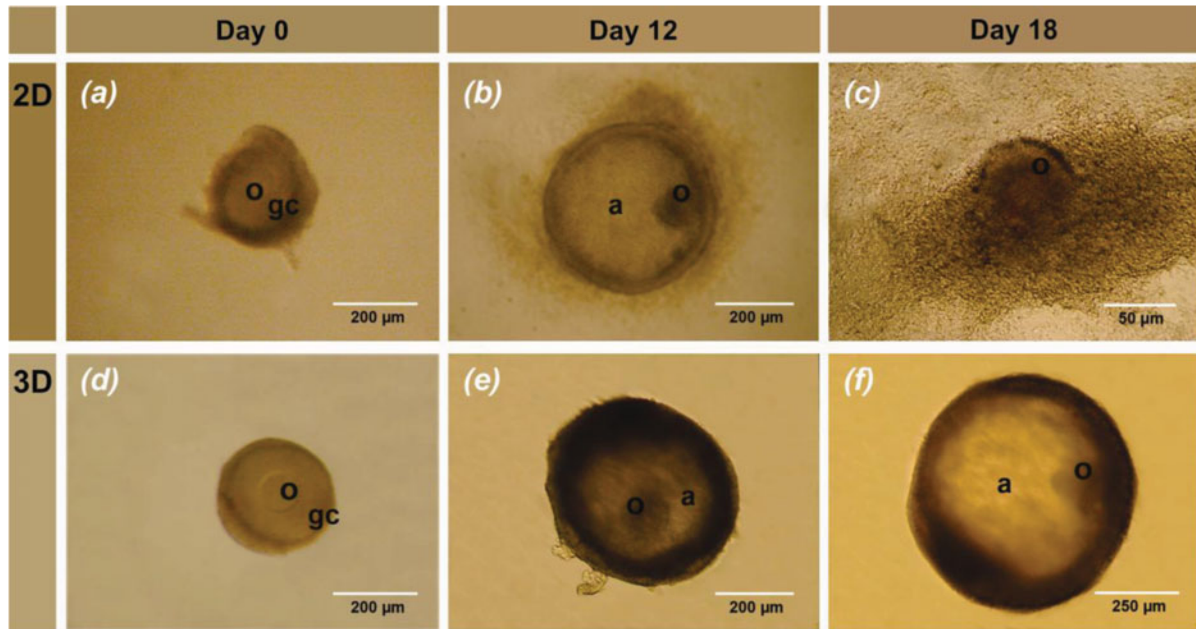

Figure 1 Adult goat ovarian follicles before culture (*a*, *d*) or cultured *in vitro* for 12 and 18 days on the two-dimensional (2D) system (*b*, *c*) or in the three-dimensional (3D) alginate culture system (*e*, *f*). o = oocyte; gc = granulosa cells; a = antrum.

Table 1 Follicular diameter (μm) of pre-antral follicles isolated from pre-pubertal (PP) or adult (AD) goats cultured *in vitro* in a two-dimensional (2D) or three-dimensional (3D) alginate system

		Follicular diameter (n)			
Goats	Follicle number	Day 0	Day 6	Day 12	Day 18
PP/2D	34	196.73 ± 7.10 (34)$^{\alpha,C}$	344.17 ± 14.43 (24)$^{\alpha,B}$	397.96 ± 21.13 (14)$^{\alpha,A}$	456.63 ± 12.76 (2)$^{\alpha,\beta,A}$
PP/3D	49	190.44 ± 5.12 (49)$^{\alpha,B}$	317.19 ± 10.47 (47)$^{\alpha,A}$	321.96 ± 11.91(38)$^{\beta,A}$	336.73 ± 20.22 (14)$^{\beta,A}$
AD/2D	37	182.16 ± 6.32 (37)$^{\alpha,C}$	332.08 ± 17.32 (23)$^{\alpha,B}$	401.2 ± 28.72 (11)$^{\alpha,A,B}$	557.82 ± 125.82 (3)$^{\alpha,A}$
AD/3D	52	194.27 ± 5.04 (52)$^{\alpha,B}$	311.12 ± 8.64 (50)$^{\alpha,A}$	336.76 ± 12.77 (43)$^{\beta,A}$	358.07 ± 21.27 (22)$^{\beta,A}$

Different letters in superscript following values indicate statistical significance among treatments in the same column ($^{\alpha,\beta}$) or among days in the same row (^{A,B,C}) ($P < 0.05$).

Table 2 Survival (%) and oocyte extrusion (%) of pre-antral follicles isolated from pre-pubertal (PP) or adult (AD) goats cultured *in vitro* in a two-dimensional (2D) or three-dimensional (3D) alginate system

		Survival % (n)				Oocyte extrusion % (n)		
Goat	Follicle number	Day 0	Day 6	Day 12	Day18	Day 6	Day 12	Day 18
PP/2D	34	100 (34)$^{\alpha,A}$	82 (28)$^{\beta,B}$	74 (25)$^{\beta,B}$	65 (22)$^{\alpha,\beta,B}$	12 (4)$^{\alpha,C}$	32 (11)$^{\alpha,\beta,B}$	59 (20)$^{\alpha,A}$
PP/3D	49	100 (49)$^{\alpha,A}$	98 (48)$^{\alpha,A}$	94 (46)$^{\alpha,A}$	65 (32)$^{\alpha,\beta,B}$	2 (1)$^{\alpha,C}$	16 (8)$^{\beta,\gamma,B}$	37 (18)$^{\beta,A}$
AD/2D	37	100 (37)$^{\alpha,A}$	73 (27)$^{\beta,B}$	65 (24)$^{\beta,B}$	51 (19)$^{\beta,B}$	11 (4)$^{\alpha,B}$	35 (13)$^{\alpha,A}$	46 (17)$^{\alpha,\beta,A}$
AD/3D	52	100 (52)$^{\alpha,A}$	98 (51)$^{\alpha,A,B}$	90 (47)$^{\alpha,B}$	75 (39)$^{\alpha,B,C}$	2 (1)$^{\alpha,B}$	8 (4)$^{\gamma,B}$	33 (17)$^{\beta,A}$

Different letters in superscript following values indicate statistical significance among treatments in the same column ($^{\alpha,\beta,\gamma}$) or among days in the same row (^{A,B,C}) ($P < 0.05$).

in survival of follicles (from both adult and pre-pubertal animals) on 2D culture by day 6 of culture ($P < 0.05$); in the 3D alginate system, survival dropped after 12 days of adult follicle culture and 18 days of pre-pubertal follicle culture (Table 2).

Except for pre-pubertal follicles on day 18 of culture, follicle survival was higher in the 3D alginate culture system compared with the 2D culture system ($P < 0.05$). No significant differences in follicle survival were observed between

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Table 3 Mean oocyte diameter, oocyte recovery rate (oocyte > 110 μm), percentage of oocytes with a germinal vesicle or evidence of meiotic resumption, the oocyte chromatin configuration and embryo production from pre-antral ovarian follicles isolated from pre-pubertal (PP) or adult (AD) goats after *in vitro* culture in a two-dimensional (2D) or three-dimensional (3D) alginate culture system for 18 days

							Embryos (*n*)	
Treatments	Oocyte diameter (μm)	Oocyte recovery % (*n*)	Germinal vesicle % (*n*)	Meiotic resumption % (*n*)	GVBD % (*n*)	MII % (*n*)	2-cell	8-cell
PP/2D	$119.86 \pm 2.41^{\beta}$	65 (22/34)$^{\alpha,\beta}$	73 (16/22)$^{\alpha}$	27 (6/22)$^{\beta}$	27 (6/22)	0 (0/22)	0	0
PP/3D	$121.80 \pm 2.87^{\beta}$	65 (32/49)$^{\alpha,\beta}$	75 (24/32)$^{\alpha}$	25 (8/32)$^{\beta}$	25 (8/32)	0 (0/32)	0	0
AD/2D	$135.75 \pm 1.63^{\alpha}$	51 (19/37)$^{\beta}$	16 (3/19)$^{\beta}$	84 (16/19)$^{\alpha}$	58 (11/19)	26 (5/19)	4	0
AD/3D	$122.07 \pm 2.28^{\beta}$	75 (39/52)$^{\alpha}$	59 (23/39)$^{\alpha}$	41 (16/39)$^{\beta}$	38 (15/39)	3 (1/39)	1	1

Different superscript letters indicate statistical significance among treatments in the same column ($^{\alpha,\beta}$) ($P < 0.05$).

pre-pubertal and adult follicles in either culture system (Table 2).

Antrum cavity formation and oocyte extrusion

The follicles of each group started to form an antrum by day 6 and there were no significant differences among the groups throughout the culture period. The antrum formation on the 18th day of culture was 47, 49, 43 and 46% for PP/2D, PP/3D, AD/2D and AD/3D (data not shown).

For all groups, the percentage of extruded follicles increased significantly from day 6 to day 18 of culture. Starting on day 12, a higher percentage of extruded follicles was observed for follicles cultured in the 2D system compared with the follicles cultured in 3D alginate. In the adult group on day 18 no difference in oocyte extrusion rates was recorded between the 2D and 3D culture systems (Table 2).

Growth, recovery rate, and chromatin configuration of oocytes

Values related to mean oocyte diameter, recovery rate, oocyte chromatin configuration and embryo production are described (Table 3). After 18 days of culture, adult follicles cultured on 2D system showed a higher mean oocyte diameter ($P < 0.05$) than the other groups (Table 3). In contrast, the oocyte recovery rate was significantly higher for adult follicles cultured in 3D alginate compared with adult follicles cultured on 2D system, but not significantly different from pre-pubertal follicles cultured on either 2D or 3D alginate. The percentage of oocytes that showed meiotic resumption was higher for oocytes from adult follicles grown on 2D culture compared with the other groups ($P < 0.05$). The cell viability assay was used to assess the integrity of non-cleaved oocyte after 2 days of embryo culture. All oocytes (PP/2D = 22 oocytes; PP/3D = 32 oocytes; AD/2D = 19 oocytes; AD/3D = 39 oocytes) were confirmed to be viable (Fig. 2*b*, *e*, *h*, *k*). The chromatin configuration of non-cleaved oocytes after day 2 of embryo culture are shown (Fig. 2*c*, *f*, *i*, *l*).

Representative images of expanded COCs after *IVM* (Fig. 3*a*) and 2-cell and 8-cell embryos after IVF of oocytes from adult follicles grown *in vitro* for 18 days on 2D culture (Fig. 3*b*) or 3D alginate (Fig. 3*c*) are shown. Only follicles isolated from adults were able to produce metaphase II oocytes (2D, $n = 5$ and 3D alginate, $n = 1$; Table 2) and 2-cell ($n = 4$), and 8-cell ($n = 1$) embryos, respectively (Fig. 3*b*, *c*).

Steroid production *in vitro*

The estradiol concentrations increased significantly from day 2 to day 12 in all groups and were similar among the groups during the entire culture period (Fig. 4*a*). Conversely, in the adult groups, progesterone production was lower in follicles cultured in 3D alginate compared with those on 2D culture, with significant differences between the groups on culture days 2 and 6 (Fig. 4*b*).

Discussion

This study was the first to compare 2D and 3D alginate culture systems for *in vitro* culture of caprine pre-antral follicles, as well as their effects on *in vitro* follicle development, oocyte maturation, and embryo production.

First, we found that follicles isolated from adult animals survived better when they were in 3D alginate than in the plastic surface (75% versus 51%). These higher survival rates reflect the ability of the 3D alginate culture system to maintain follicular morphology. Previous studies have found that the encapsulated 3D follicle culture systems may overcome the technical challenge of maintaining follicle architecture during the longer term cultures needed for larger species (Ksiazkiewicz, 2006). Moreover, in mice, the preservation of the 3D follicular morphology

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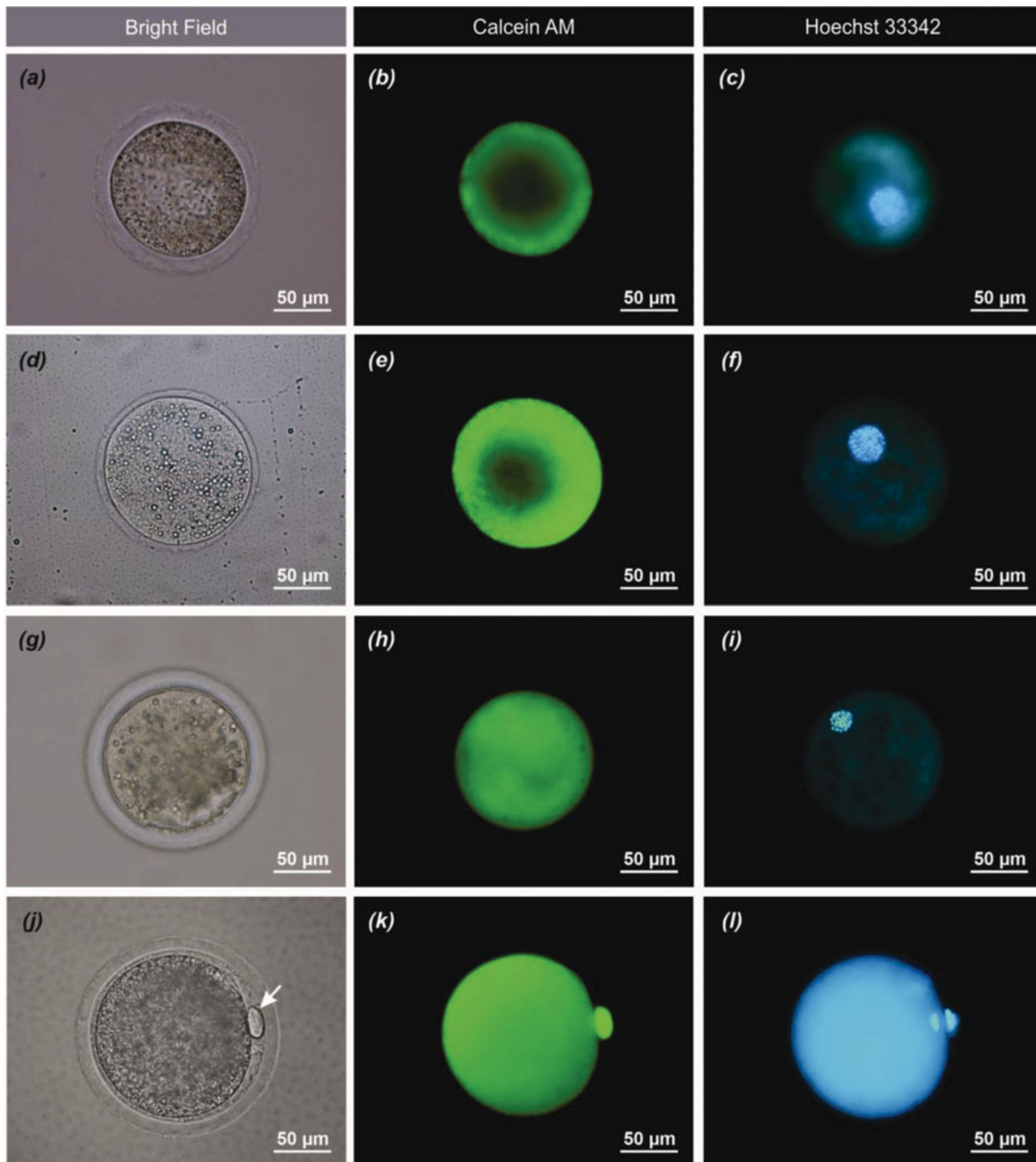

Figure 2 Viability and chromatin configuration of representative oocytes chosen for *in vitro* maturation (IVM) after *in vitro* culture of adult goat pre-antral follicles. Oocyte visualization in bright field (*a*, *d*, *g*, *j*). The arrow indicates the first polar body (*j*). Characterization of a viable oocyte after staining with calcein-AM (*b*, *e*, *h*, *k*) and after staining with Hoechst 33342 (*c*, *f*, *i*, *l*). Hoechst 33342 staining under fluorescence shows oocytes with a germinal vesicle (*c*), germinal vesicle breakdown (*f*, *i*) and in metaphase II (*l*).

promotes an appropriate culture environment. This approach represents a promising system to optimize the follicular *in vitro* culture systems (Vanhoutte *et al.*, 2009).

We also noted that alginate seemed to act as a barrier to follicle growth, with the rate of growth slowing (smaller increases in diameter) after 6 days of culture. Previous studies have demonstrated that encapsulation of mouse follicles in alginate at 1.5% w/v resulted in a slow rate of follicle growth after 8 days of culture. In the same species, extension of the culture period to 12 days and reduction in the alginate concentration to 0.25% and 0.5% led to an increase in follicle diameters up to the end of culture (Xu *et al.*, 2006a). It is likely that in our cultures of caprine follicles, the alginate concentration

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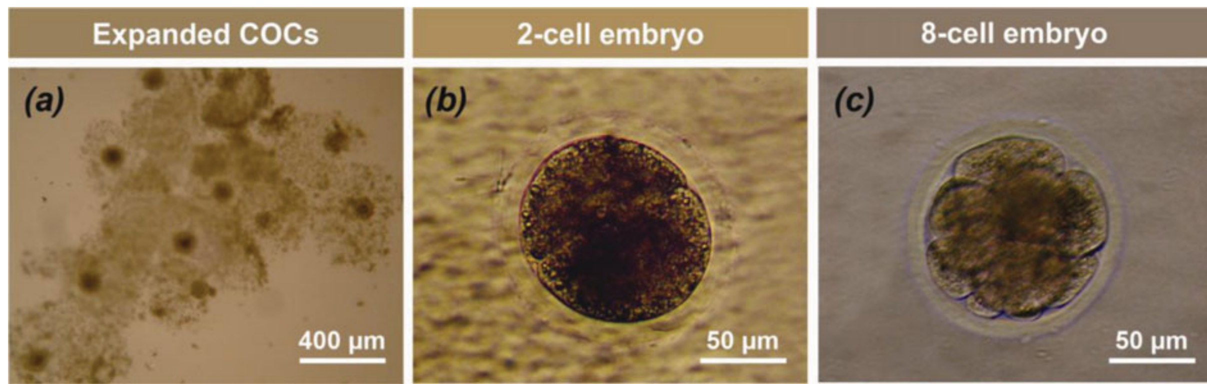

Figure 3 Representative images of expanded COCs after *in vitro* maturation (*a*) and 2-cell and 8-cell embryos after IVF of oocytes from adult follicles cultured on two-dimensional (2D) (*b*) or three-dimensional (3D) alginate (*c*) and for 18 days.

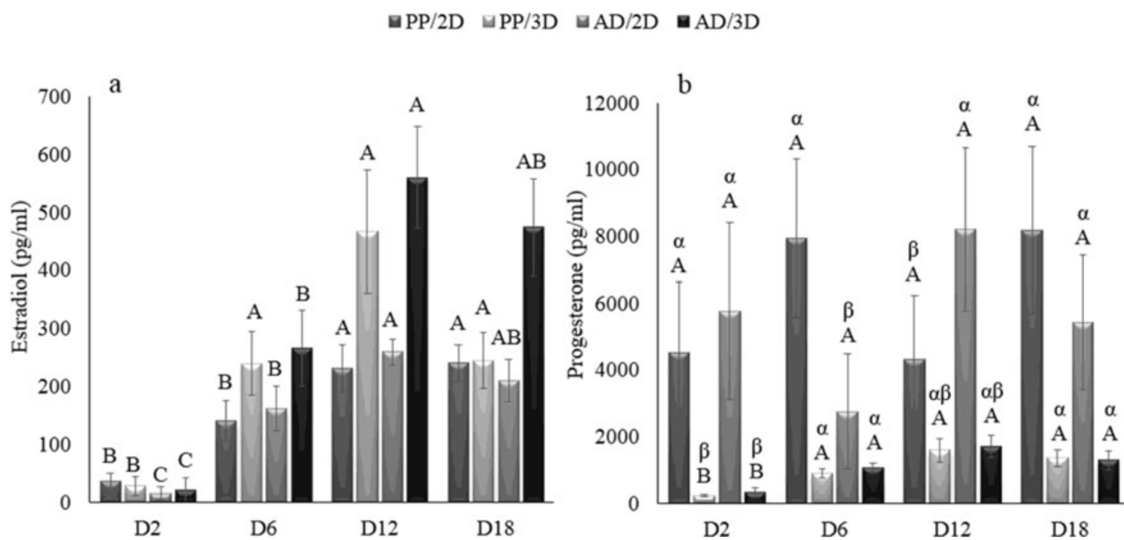

Figure 4 Estradiol (*a*) and progesterone (*b*) secretion by ovarian pre-antral follicles isolated from pre-pubertal or adult goats and cultured on either in two-dimensional (2D) or in three-dimensional (3D) alginate for 2, 6, 12, or 18 days. Different letters indicate statistical significance among treatments on the same day ($^{\alpha,\beta}$) or among days with the same treatment (^{A,B,C}) ($P < 0.05$).

or length of culture was not ideal to support follicle expansion. Nevertheless, because we achieved good caprine follicle survival in the 3D alginate culture system, we will be able to extend the culture period to test this hypothesis in future studies.

Incorporation of other components into the alginate gel, such as collagen I and IV, fibronectin, and laminin, has been shown to support follicular development *in vitro*. Other studies have combined alginate with fibrin, a protein involved in blood coagulation, to produce an innovative biomaterial that can accommodate the outward growth of the follicle (Shikanov *et al.*, 2009).

In this study, we observed no difference in the rates of antrum formation among the four groups of follicles. Our antrum formation rates were also lower than those observed in other studies of pre-antral caprine follicle culture in 2D culture systems (Araújo *et al.*, 2011). This result may be explained by our addition of fetuin to the base culture medium, which promotes follicle adhesion to the culture plate and impedes antral cavity formation and visualization. In this study, we added fetuin to the culture medium to prevent ZP hardening during culture. During spontaneous maturation of mouse oocytes in serum-free medium, the ZP becomes 'hardened' or resistant to chymotrypsin digestion (De Felici and Siracusa, 1982) as well as sperm penetration, thus preventing fertilization. This phenomenon has also been observed in non-human primate (Van de Voort *et al.*, 2007) and human (Schiewe *et al.*, 1995) oocytes. The addition of serum from various sources, including FBS, effectively prevents ZP hardening in rodents (Eppig and Schroeder, 1986). Fetuin, a major glycoprotein in serum and follicular fluid, increases the solubility of the ZP during spontaneous oocyte maturation in mice; addition of fetuin removes the need for serum and

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permits the use of a serum-free environment for follicle culture (Schroeder *et al.*, 1990). Fetuin has also been used as a cell fixation factor during *in vitro* culture in the absence of FBS (Fisher *et al.*, 1958). In the 3D alginate culture system, the restricted follicle growth we observed may also explain the reduced follicular antrum formation compared with 2D cultures.

A significant increase in oocyte extrusion was observed in all groups from day 6 to day 18 of culture, suggesting that alginate is unable to prevent oocyte extrusion. However, in general, there was a reduced tendency for oocyte extrusion during the entire culture period in the 3D alginate groups. It has been suggested that follicle rupture is related to inadequate three-dimensional architecture maintenance and other factors such as inadequate follicular basal membrane remodeling (Silva *et al.*, 2011).

In this study, the assessment of oocyte viability by fluorescence microscopy was performed only after IVF. In this evaluation it was observed that all follicles classified as morphologically normal (survivors) were really viable, i.e. their oocytes were marked by the green calcein-AM. The mean oocyte diameter and meiotic resumption rate were higher in adult follicles on 2D culture. Oocyte diameter is closely linked to the ability of oocytes to resume meiosis. Crozet *et al.* (2000) found that oocytes with a diameter greater than or equal to 110 μm have greater competence to resume meiosis and, subsequently, maturation. Although adult follicles cultured in 3D alginate produced a higher number of oocytes with a diameter greater than 110 μm, when compared with adult follicles cultured in 2D system; the highest mean oocyte diameter was observed for adult follicles on 2D culture. This situation may explain the higher meiotic resumption rates observed in the adult follicle group grown on 2D culture compared with either the pre-pubertal follicle groups or the adult follicle group cultured in 3D alginate.

Estradiol and progesterone secretion patterns among the four groups of cultured follicles suggest that the physiological function of the follicle cells and interactions between the oocyte and granulosa cell/theca cells were maintained (Adriaens *et al.*, 2004). Estradiol production increased after 12 days of culture in all groups, indicating that steroidogenic thecal cell function was preserved. Our observation that the 3D alginate culture system produces the lowest level of progesterone at 2 and 6 days of culture in the adult group when compared with adult 2D group indicates no premature luteinization of follicular cells. Thus, the 3D alginate system supported normal follicle development.

The reproductive age of the ovarian follicle donor (pre-pubertal versus adult) did not affect follicle survival, follicle diameter, antrum formation, or oocyte extrusion rates. However, adult follicles on 2D culture achieved significantly larger oocyte diameters and higher meiotic resumption rates compared with pre-pubertal follicles cultured on the same system (2D). In addition, adult follicles, regardless of culture system, were the only follicles able to produce metaphase II oocytes and embryos. Our data suggest that the reproductive age of the ovarian donor does affect the *in vitro* oocyte growths at the pre-antral follicle stage and the meiotic competence consequently. To increase the rates of oocyte maturation, the culture of follicles from pre-pubertal animals may require supplements to the culture medium or special culture conditions that are not needed for follicles from adult animals.

In conclusion, the oocyte resumption rates was improved by the 2D culture system using follicles from adult animals. Furthermore, although the culture of pre-antral caprine follicles on 2D system resulted in greater oocyte diameters and meiotic resumption rates, it can be suggested that 3D alginate system may support better survival, resulting in higher oocyte recovery for IVM.

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