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Development of goat primordial follicles after in vitro culture of ovarian tissue in Minimal Essential Medium supplemented with coconut water

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Abstract

In the development of culture systems for primordial follicles is important for studying their growth and differentiation *in vitro*. In this study, we investigated the effect of minimal essential medium (MEM), coconut water solution (CWS), and MEM supplemented with CWS on the morphology and activation of primordial goat follicles. Ovarian, cortical tissue pieces were cultured for 1 or 5 days in MEM, CWS, or MEM supplemented with 5, 10, 20, 50, 80, 90, or 95% CWS. Both MEM and CWS were supplemented with BSA, ITS, glutamine, pyruvate, and hypoxanthine. On day 0 and after 1 or 5 days of *in vitro* culture, the cortical pieces were fixed for histological evaluation. Based on their morphology, follicles were classified as primordial, developing and intact, or atretic. Follicular diameter was evaluated before and after culture. Mitotic activity of granulosa cells was studied by immunolocalization of proliferation cell nuclear antigen (PCNA). The results showed that the percentage of primordial follicles was reduced ($P < 0.05$) and was concomitant with the increase of the percentage of developing follicles in all media tested after 5 days culture. The highest rate of primordial follicle activation was observed after 5 days of culture when ovarian pieces had been cultured in MEM or MEM supplemented with 5 or 10% CWS. However, culturing follicles for 5 days in a mixture of MEM and 20% or higher percentages of CWS reduced their activation and viability. In all media tested, mean follicular diameters had significantly increased after 5 days of culture when compared to those measured at day 0 and day 1. Immunohistochemical analysis showed that primordial follicles generally do not stain for PCNA both in non-cultured and cultured tissues, whereas the granulosa cells of developing follicles do express PCNA after culture. In conclusion, goat primordial follicles were activated and kept viable after *in vitro* culture in MEM or in a mixture of MEM with low proportions (10% or less) of CWS. Compared to MEM alone, addition of CWS to MEM does not improve the survival, activation, or further development of the primordial follicle population during culturing of goat ovarian cortical pieces.

Keywords: goat, ovary, primordial follicles, coconut water

Introduction

Primordial follicles begin to develop during childhood and the number of primordial follicles is established after birth (Cha *et al*., 2000). However, of the thousands of primordial follicles present at birth, the vast majority (99.9%) become atretic during growth and maturation and only a very small number of follicles achieve maturity and ovulate (Baker, 1963). Thus, the study of early folliculogenesis as well as the development of *in vitro* conditions to promote primordial follicle development maybe important to reduce follicular demise that occurs *in vivo*. Better knowledge of culture conditions is also very important for the study of factors involved in the activation and further growth of primordial follicles. Optimal *in vitro* culture conditions may lead to the generation of large numbers of healthy oocytes that can be used for *in vitro* fertilization (IVF) procedures, intracytoplasmic sperm injection (ICSI), and cloning.

Over the last two decades, many culture systems have been developed with the aim to promote activation and growth of primordial follicles *in vitro*, although knowledge about the factors that control the earliest stages of folliculogenesis is still scarce. During the onset of follicle growth, the enclosed oocyte begins to grow and the flattened pre-granulosa cells of primordial follicles becomes cuboidal and begins to proliferate (Hirshfield, 1991). The success of studies on the initiation of follicular development is generally assessed by detecting the onset of follicular growth by the availability of sensitive markers, like the proliferating cell nuclear antigen (PCNA; Liu *et al*., 1989). Several studies showed that expression of PCNA is correlated with the initiation of follicular growth in humans (Oktay *et al*. 1998; 2000), in the non-human primates (Gougeon and Busso, 2000), cows (Wandji *et al*., 1996; Fricke *et al*., 1997), sheep (Lund *et al*., 1999), mice (Oktay *et al*., 1995), and goats (Silva *et al*., 2004).

When studying primordial follicle activation *in*

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vitro, the type of medium used has a significant influence on follicular survival and growth. Minimal essential medium has been used for culturing bovine (Braw-Tal and Yossefi, 1997) and caprine (Silva *et al*., 2004) ovarian tissue and isolated bovine (Figueiredo *et al*., 1994) and murine (Zhao *et al*., 2001) preantral follicles. Coconut water solution (CWS) is a natural solution, inexpensive, and rich in nutrients, like proteins, sugars, vitamins, salt, neutral lipids (Marques, 1982), and substances that can induce cellular division and electrolytes that promote the survival and viability of cryopreserved male and female gametes (Blume and Marques, 1994). Indole-3-acetic acid (IAA), an important molecule present in coconut water, belongs to an auxins group and has a beneficial effect on the metabolism of goat sperm thus increasing motility and fertility rates and allowing a long duration of preservation (Nunes and Combarnous, 1995). In India, substances that promote growth in plants such as endogen cytokines and zeatine ibozide have been isolated from coconut water (Nunes and Salles, 1993). Silva *et al.* (2004) suggested that IAA can bind to certain animal growth factors present in the ovarian tissue, modulating the action of these growth factors.

Coconut water solution has successfully been used for murine oocyte (Blume et al., 1997a) and embryo culture (Blume et al., 1997b), as well as for culture and preservation of goat preantral follicles *in vitro* (Silva *et al*., 2000; 2004; Andrade *et al*., 2002). Silva *et al*. (2004) demonstrated that the activity and viability of early-staged follicles cultured in MEM plus 25% CWS was similar to those cultured in MEM alone, and that follicular viability was reduced when the proportion of CWS was increased. Therefore, the effects of MEM and CWS mixtures on primordial follicles, consisting of relatively low percentages of CWS, are still unknown.

The aim of this work was to investigate the effects of MEM, CWS, or mixtures of MEM with low or high proportions of CWS on viability, activation, and further growth of goat primordial follicles cultured for 5 days. Additionally, immunohistochemical localization of proliferating cell nuclear antigen was performed to evaluate granulosa cell proliferation.

Materials and Methods

Ovaries

Ovaries (n=10) from five mixed-breed goats were collected at a local slaughterhouse. Immediately postmortem, the ovaries were washed in 70% alcohol for 10 seconds following two times in 0.9% saline solution for 10 seconds. The pairs of ovaries were transported within 1 hour to the laboratory in 0.9% saline solution at 37 °C. In the laboratory, the ovaries from each animal were stripped of surrounding fat tissue and ligaments and then cut in half. The medulla, large antral follicles, and corpora lutea were removed. Subsequently, the ovarian cortex of each pair of ovaries was divided into 19 fragments of approximately 3mm x 3mm x 1mm in volume.

In vitro culture of ovarian tissue

One fragment was taken randomly and immediately fixed for histological examination (control). The 18 fragments remaining were cultured individually in a culture dishes with 24-wells, each containing 1 ml of culture medium at 39 °C with 5% CO2 in air. The media used were composed of either or both: (1) minimum essential medium (MEM – osmolarity: 300 mOsm/l, pH: 7.2 – Cultilab, Rio de Janeiro, Brazil), and (2) sterile coconut water solution composed of two parts of coconut water, one part of pure water and one part of sodium citrate 5% (final osmolarity: 300 mOsm/l and pH: 7.2). Coconut water was obtained from coconuts (6 months old) collected from the green beach variety of the coconut - palm (*Cocus nucifera*). Both media were supplemented with antibiotics (100 µg/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml fungizone), ITS (insulin 6.25 µg/ml, transferrin 6.25 µg/ml, and selenium 6.25 ng/ml), 0.23 mM pyruvate, 2 mM glutamine, 2 mM hypoxanthine, and 1.25 mg/ml BSA. The ovarian cortex fragments were cultured for 1 or 5 days in 9 different culture media as described below.

- Minimum Essential Medium (MEM)
- MEM plus 5% CWS (MEM/5% CWS)
- MEM plus 10% CWS (MEM/10% CWS)
- MEM plus 20% CWS (MEM/20% CWS)
- MEM plus 50% CWS (MEM/50% CWS)
- MEM plus 80% CWS (MEM/80% CWS)
- MEM plus 90% CWS (MEM/90% CWS)
- MEM plus 95% CWS (MEM/95% CWS)
- Coconut water solution (CWS)

Histological evaluation and assessment of granulose cell proliferation by PCNA

Immediately after fragmentation, representative pieces of ovarian cortex were immersion-fixed for 12 h in 10% neutral buffered formaldehyde (pH 6.8-7.2) to serve as non-cultured controls. After 1 or 5 days of culture in each medium, the pieces of ovarian cortex were fixed as described above, dehydrated in a graded series of ethanol, clarified with xylene, and embedded in paraffin wax. For each piece of ovarian cortex, 7 µm thick tissue sections were mounted on coated slides and stained with Periodic Acid Schiff and hematoxylin. After 5 days culture, some slides were stained with antibodies for PCNA (DAKO, Carpinteria, CA, USA) as described previously (Oktay *et al*., 1995). Briefly, the sections were paraffinized, rehydrated and treated for 8 min at 93 – 98 ºC in citric buffer at pH 6, and then incubated for 30 min at room temperature (~20 ºC) in 3% H_2O_2 for 30 min to block endogenous peroxidase activity. After washing, normal rabbit serum was added for 20 min at room temperature to inhibit non-specific binding. The first antibody was incubated at a dilution of 1:50 for 18h at 8°C followed by washing and incubation in rabbit anti-mouse IgG (DAKO, Carpinteria, CA, USA) at 1:200 dilution for 30 min. In

control sections, the primary antibody was replaced by Tris-buffered saline. Finally, streptavidin–biotin complex (DAKO, Carpinteria, CA, USA) was added for 30 min at room temperature, followed by diaminobenzidine (DAB - DAKO, Carpinteria, CA, USA) for a further 7 min. After completing the reactions, the sections were counterstained with hematoxylin and evaluated for the presence or absence of PCNA. All sections were evaluated with optical microscope (200 and 400x magnification). From each medium and after each culture period, approximately 150 follicles were randomly evaluated.

Follicle classification and measurement

The follicles were classified individually according to shape and number of granulosa cells layers around the oocyte as primordial (one layer of flattened granulosa cells) or developing follicles (one layer of flattened to cuboidal granulosa cells), primary (a single layer of cuboidal granulosa cells), or secondary (two or more layers of cuboidal granulosa cells). The percentages of primordial and developing follicles were calculated on day 0 (control) and after 1 or 5 days of culture in each medium. In addition, follicular diameter was taken using only normal follicles from day 0 and after *in vitro* culture for 1 or 5 days. Only follicles with a visible oocyte nucleus were evaluated to avoid counting the same follicle twice. Follicular quality was evaluated based on morphological parameters such as: oocyte and granulose cells morphology and basement membrane integrity (Silva *et al*., 2000). The primordial and developing follicles were classified as morphologically normal (follicles containing an intact oocyte and granulosa cells well-organized in layers without a pyknotic nucleus) and degenerated follicles (oocyte with pycnotic nucleus, retracted cytoplasm and disorganized granulosa cells detached from the basement membrane) (Silva *et al*., 2000).

Statistical analyses

The percentage of morphologically normal follicles, as well the percentage of primordial or developing follicles in non-cultured tissue or after 1 or 5 days culture in different media were compared by a Chi-squared test (Instat for Macintosh). Follicle diameters in non-cultured and cultured tissue were compared by ANOVA and the Kruskal-Wallis test. Differences were considered statistically significant at <0.05.

Results

Percentage of normal follicles in non-cultured (control) and cultured ovarian cortex

Histological analysis showed that normal (Fig. 1A) and degenerated (Fig. 1B) follicles were found in non-cultured and cultured ovarian cortical pieces. In degenerated follicles, a shrunken oocyte, a pyknotic nucleus, and disorganized granulosa cells were observed. Table 1 shows the percentage of normal follicles in non-cultured (day 0: control) ovarian cortical pieces and after 1 or 5 days of culture in different media. Culture of ovarian tissue for 1 or 5 days, in all media tested, significantly reduced ($P<0.05$) the percentage of intact follicles when compared to non-cultured cortical tissue. The increase of culture duration from 1 to 5 days significantly reduced the percentage of intact follicles observed in tissue cultured in MEM plus 50, 80, and 90% CWS. After 1 day of culture, a progressive and significant reduction in the percentage of normal follicles was observed in MEM containing increasing proportions of CWS from 0 to 10, 50, 80, and 95%. After 5 days of culture, MEM, MEM plus 5 and 10% CWS showed a significantly higher percentage of normal follicles when compared to the other media, except between MEM plus 5% CWS and MEM plus 20% CWS. The percentage of intact follicles between MEM 80, 90, 95% CWS and CWS was not different.

Goat primordial follicle activation in vitro

The percentage of primordial and developing follicles in non-cultured ovarian cortex was 69.2 and 30.8%, respectively (Fig. 2). After 1 day of culture, the percentage of primordial or developing follicles did not differ significantly among the tested media or when compared to day 0. In contrast, after 5 days of culture in all media tested, the percentage of primordial follicles was reduced ($P<0.05$), and there was a concomitant increase ($P<0.05$) in the percentage of developing follicles compared to non-cultured tissue. Cortical tissue cultured in MEM for 5 days had a higher percentage ($P<0.05$) of developing follicles when compared to MEM plus 20, 50, 80, 90, 95% CWS and pure CWS; however, no significant differences were found among MEM and MEM plus 5 and 10% CWS. The percentage of developing follicles cultured in MEM plus 5, 10, 20, 50, 80, 90, 95% CWS and CWS did not differ from each other, except MEM plus 5% CWS had a higher percentage ($P<0.05$) of developing follicles than MEM plus 80% CWS.

Follicle diameter in non-cultured and cultured tissue

Table 2 shows follicle diameter in non-cultured ovarian cortex and after *in vitro* culture for 1 or 5 days. After 5 days of culture, in all media, a significant increase in follicle diameter was found when compared to follicles in non-cultured tissue or in tissue cultured for 1 day. Independent of culture period (1 and 5 days), no significant effect of media composition on follicular diameter was found.

Effect of in vitro culture on granulosa cell proliferation

In non-cultured ovarian cortex, PCNA staining was generally absent in granulosa cells of primordial

follicles (Fig. 3A) and only a few developing follicles had PCNA positive granulosa cells. After 5 days of culture, independent of the culture medium used, the majority of follicles were developing and had intense PCNA immunostaining in granulosa cells (Fig. 3B and C). Occasionally, PCNA immunoreactivity was present in oocytes of both primordial and developing follicles (Fig. 3).

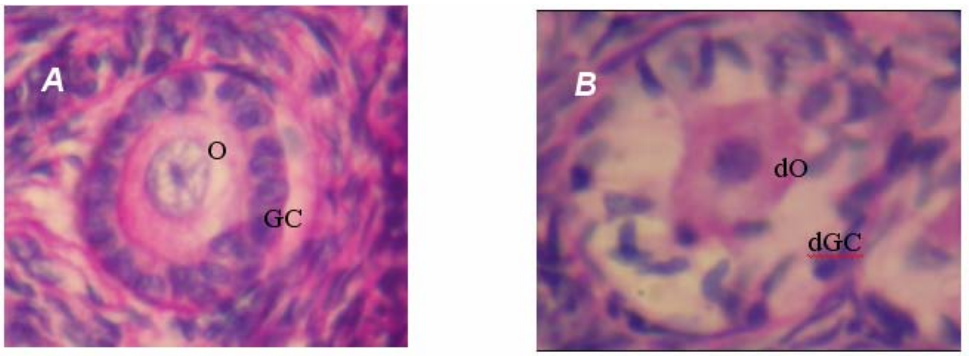

Figure 1. Histological section of a (A) normal and (B) degenerated follicle after staining with PAS-hematoxylin. O: oocyte, GC: granulosa cells, dO: degenerated oocyte, dGC: degenerated granulosa cells (400x magnification).

Table 1. Percentage of normal follicles in non-cultured tissue and in tissue after culture for 1 or 5 days in different media.

Non-cultured	83.6 % (107/128)			
Cultured	Day 1		Day 5	
MEM	81.1% $^{A, a}$	(99/122)	68.2% * $^{B, a}$	(88/129)
MEM/5% CWS	74.2% * $^{A, ab}$	(89/120)	66.9% * $^{A, ab}$	(87/130)
MEM/10% CWS	68.3% * ^{A,bc}	(82/120)	67.8% * $^{A, a}$	(80/118)
MEM/20% CWS	60.0% * $^{A, cd}$	(75/125)	56.9% * $^{A, b}$	(70/123)
MEM/50% CWS	54.8% * $^{A, d}$	(68/124)	38.4% * $^{B, c}$	(48/125)
MEM/80% CWS	41.7% * $^{A, e}$	(48/115)	24.1% * $^{B, d}$	(28/116)
MEM/90% CWS	38.4% * $^{A, e}$	(43/112)	20.2% * $^{B, d}$	(23/114)
MEM/95% CWS	23.5% * $^{A, f}$	(28/119)	15.7% * $^{A, d}$	(20/127)
CWS	22.3% * $^{A, f}$	(27/121)	17.7% * $^{A, d}$	(22/124)

*Differs significantly from non-cultured tissue (control).
^{A,B} – Values with different letters in the same row show are significantly differences (P<0.05).
^{a,b,c,d,e,f} – Values within columns with different letters among media compositions are significantly different (P<0.05).

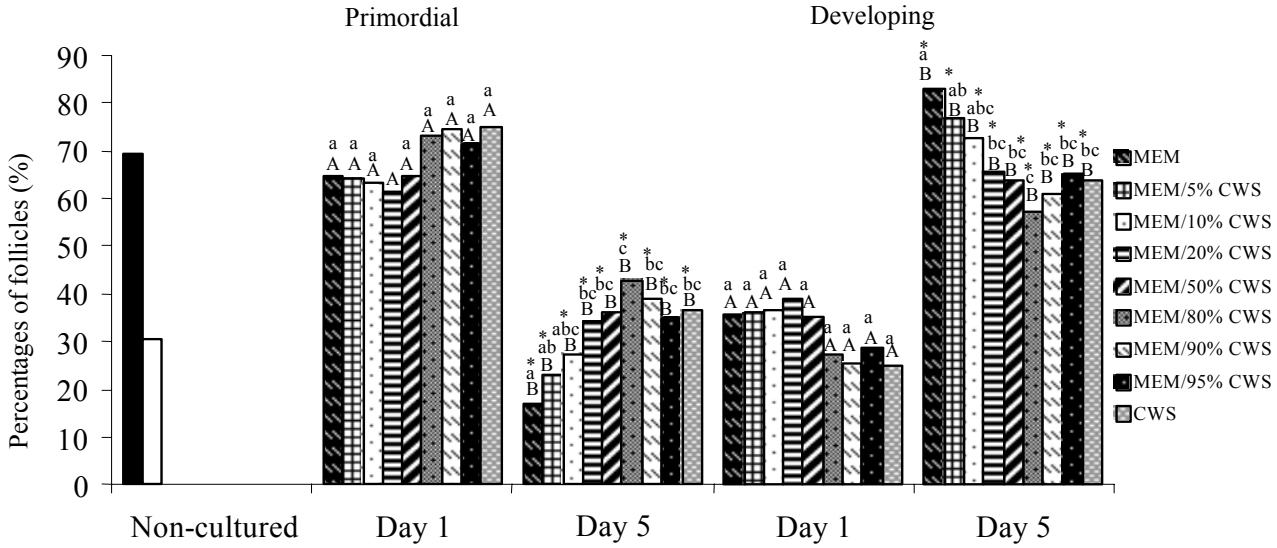

Figure 2. Percentage of primordial (■) and developing (□) follicles in non-cultured tissue and in tissue after culture for 1 or 5 days in different media. (*) Denotes values that differ significantly from non-cultured tissue (control). A, B - Values with different letters are significantly different between culture periods. a, b, c - Values with different letters among media compositions are significantly different.

Table 2. Mean follicle diameter (μm ± SD) in non-cultured ovarian cortex and after *in vitro* culture for 1 or 5 days.

Non-cultured	50.2 ± 7.2	
Cultured	Day 1	Day 5
MEM	49.4 ± 8.1 ^{A}	59.0 ± 8.5 * ^{B}
MEM/5% CWS	49.4 ± 8.0 ^{A}	58.4 ± 8.8 * ^{B}
MEM/10% CWS	49.0 ± 7.6 ^{A}	54.6 ± 8.7 * ^{B}
MEM/20% CWS	48.0 ± 7.6 ^{A}	54.8 ± 6.0 * ^{B}
MEM/50% CWS	50.9 ± 7.6 ^{A}	60.5 ± 9.0 * ^{B}
MEM/80% CWS	52.7 ± 6.1 ^{A}	58.2 ± 9.4 * ^{B}
MEM/90% CWS	53.7 ± 8.3 ^{A}	60.0 ±10.0 * ^{B}
MEM/95% CWS	53.0 ± 7.8 ^{A}	60.5 ± 7.6 * ^{B}
CWS	53.9 ± 7.9 ^{A}	61.8 ± 9.4 * ^{B}

*Differs significantly from non-cultured tissue (control).
$^{A, B}$ – Values with different letters within the same row are significantly different (P<0.05).

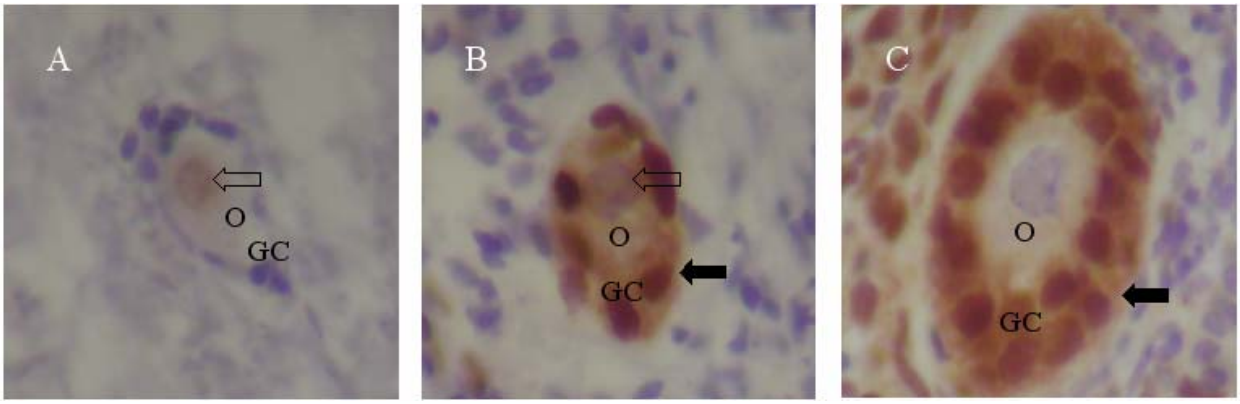

Figure 3 – Sections of ovarian tissue showing (A) primordial follicles on day 0 and (B and C) developing follicles after 5 days culture. PCNA staining in oocyte (⇦)and granulosa cells (⬅). O: oocyte, GC: granulosa cells (400x magnification).

Discussion

This study showed that goat primordial follicles can be activated after *in vitro* culture in MEM, CWS, or in mixtures of MEM and CWS, and the percentages of developing and viable follicles decrease with increasing proportions of CWS mixed with MEM. After 1 day of culture, the percentages of primordial and developing follicles were similar to control values. However, after 5 days, in all media types, a significant reduction in the percentage of primordial follicles with a concomitant increase in the percentage of developing follicles was found. Similar results were obtained in studies with bovines (Wandji *et al*, 1996; Braw-Tal and Youssefi, 1997) and baboon (Fortune *et al*., 1998) ovarian sections in which the number of primordial follicles was dramatically reduced with a concomitant increase in the number of developing follicles after 2 days of culture. Possibly, the release of stimulatory factors or cessation of production of inhibitory factors by stromal, granulosa, or pre-thecal cells within the culture of ovarian cortical tissue triggered the *in vitro* activation of the goat primordial follicles. In this respect, studies have demonstrated that anti-Mullerian hormone (Durlinger *et al*., 1999) and IGF-1 (Fortune *et al*., 2004) can inhibit but not block the activation of primordial follicles *in vitro*. The main source of these hormones are the granulosa cells of more advanced follicles. Low concentrations of such inhibitory factors in the cultured cortical pieces or the presence of possible stimulatory factors in the culture media, such as insulin (Kezele *et al*., 2002) may explain the observed primordial follicle activation *in vitro*. Locally produced growth factors that stimulate primordial follicle activation are BMP-7, Kit Ligand, GDF-9, and FGF-2 (Fortune, 2003; Van den Hurk and Zhao, 2005).

In this study, after 5 days of culture, the supplementation of MEM with 5 and 10% of CWS kept the percentage of activated follicles similar to that of tissue cultured in MEM; whereas the use of 20% or higher proportions of CWS and pure CWS decreased the percentage of developing follicles. In a report from from our group (Silva *et al*., 2004), additon of more than 25% CWS to MEM reduced the number of developing follicles. The percentage of degenerated follicles was lower than in the current study and significantly increased with use of pure CWS. The disagreements between the current study and that

described by Silva *et al*. (2004) could be due to differences in the quality of the follicles or variation in the composition of the coconut water used because of the natural source. The coconuts were derived from the same tree however. Various authors concluded that coconut water solution could be successfully used for *in vitro* culture of preantral follicle from goat (Silva *et al*., 2004), sheep (Andrade *et al*., 2002), and in dog semen preservation *in vitro* (Cardoso *et al*., 2002). In others studies, coconut water was as efficient as TCM 199 for bovine oocyte maturation (Blume *et al*., 1997a) and embryo culture (Blume *et al*., 1997b). Satisfactory results using coconut water were ascribed to the many nutrients (Laguna and Nunes, 1997; Santoso *et al*., 1996) and antioxidants in this fluid (Leong and Shui, 2002). Leong and Shui (2002) showed that ascorbic acid, present in CWS, contributes largely to the antioxidant activity of CWS. Based on our findings, we concluded that CWS negatively influenced follicular development dependent of the amount added, and therefore dissuade~~s~~ the use of CWS, either pure or in a mixture with MEM, as a culture medium for early-staged, goat follicles.

The percentage of morphologically normal follicles in non-cultured tissue was similar to those described previously for goats (Silva *et al*., 2001; Carvalho *et al*., 2001). After 1 day of culture, MEM was the only medium that kept the percentage of normal follicles at the level of that in control tissue. MEM is widely used to culture bovine preantral follicles *in vitro* (Hulshof *et al*., 1995; Katska and Rinska, 1998; Figueiredo *et al*., 1994; Saha *et al*., 2000). Jewgenow (1998) reported that MEM is more effective than TCM 199 to keep the viability of cat preantral follicles *in vitro*. The supplements added to MEM contributed to the survival of early-staged follicles from goats (Silva *et al*., 2004), cats (Jewgenow, 1998) and cows in conjunction with *in vitro* culture (Figueiredo *et al*., 1994). After 5 days of culture, results showed that CWS reduced ~~the~~ follicular viability~~,~~ when added at increasing proportions. Silva *et al*. (2004) also demonstrated a reduction in the percentage of normal follicles after culture in medium containing CWS. Despite being successfully used to culture oocytes (Blume *et al*., 1997a) and to preserve semen (Cardoso *et al*., 2002)~~,~~ when compared to MEM, CWS did not improve the survival of early-staged, goat follicles cultured *in vitro* and even had an atrophic effect on these follicles when added to MEM in higher proportions or as pure CWS.

The present data show that, regardless of the tested medium composition, goat primordial follicles were able to grow during 5 days of *in vitro* culture. Our findings are in agreement with those of Hreinsson *et al*. (2002)~~,~~ who showed that the diameter of early-staged, human follicles increased significantly after 7 days of culture. Studies of Wandji *et al*. (1996) indicated that the diameter of normal primordial and developing follicles during *in vitro* culture had significantly increased after 2 days. Thomas *et al*. (2003) showed that, after 6 days of culture, there was a significant increase in the diameter of ovine follicles. In the baboon, the increase in the diameter of ovarian follicles may be due to oocyte growth and granulosa cells proliferation (Wandji *et al*., 1997). Indeed, the increase of follicular diameter after 5 days of culture corresponded to the wide-spread PCNA immunorectivity in granulosa cells and oocytes of developing follicles. The PCNA staining of granulosa cells was indicative of granulosa cell proliferation. PCNA staining in oocytes may be due to DNA repair (Downey *et al*., 1990) during the intense RNA transcription that occurs in growing oocytes (Lintern-Moore and Moore, 1979). In agreement with our findings in goat follicles, the onset of bovine primordial follicle growth was also accompanied by PCNA expression in granulosa cells and oocytes (Wandji *et al*., 1996).

In summary, this study showed that goat primordial follicles are activated *in vitro* in MEM, CWS or mixed solutions composed of MEM in different proportions of CWS. Culture in mixtures of 5 or 10% CWS and MEM kept these latter paramenters in similar levels when compared to those obtained by MEM. However, when compared to the effect of MEM alone, addition of CWS to MEM did not improve follicular viability, activation, or growth. In proportions of 20% or higher, CWS negatively influenced early follicular quality. It is concluded that, when used as culture media for goat ovarian primordial follicles, pure CWS or mixtures of CWS and MEM, compared to MEM alone, do not improve early-staged follicle survival, activation, or further growth.

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