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In vitro culture of ovine primordial follicles in media supplemented with coconut water

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Abstract

We investigated the effect of minimal essential medium (MEM), coconut water solution (CWS), and MEM supplemented with CWS on viability, activation, and growth of ovine primordial follicles. Ovarian cortical slices were cultured *in vitro* for 1, 3, 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 FCS, ITS, glutamine, pyruvate, and hypoxanthine. Before (Day 0) and after 1, 3, or 5 days of *in vitro* culture, follicles were isolated from the ovarian tissue, and their diameters were measured as well as evaluated by using trypan blue staining. Based on the uptake or not of trypan blue, follicles were classified as viable or nonviable, respectively. Follicles were also classified as primordial or developing based on their morphology. The results showed a reduction ($P < 0.05$) in viable follicles after 1 day of culture compared to uncultured controls with the exception of MEM supplemented with up to 20% CWS. After 5 days of culture, significantly higher percentages of viable follicles were observed in MEM and MEM plus 5% CWS. A percentages of developing follicles increased significantly after culturing cortical tissue for 5 days in MEM or MEM plus 5 or 10% CWS. In all media tested, a significant increase in follicle diameter was observed after 5 days of culture with MEM only or MEM with 10% CWS. In conclusion, sheep primordial follicles are successfully activated *in vitro* after culture in MEM alone or with 5 or 10% CWS.

Keywords: sheep, primordial follicles, *in vitro* culture, MEM, coconut water.

Introduction

The ovary of sheep (Cahill, 1981) as well as humans (Oktay *et al*., 1997) contains mostly primordial follicles with one layer of flat granulosa cells surrounding the oocyte in newborns as well as adults. Developing follicles are defined in this manuscript as either primary (one layer of cuboidal granulosa cells surrounding the oocyte) or secondary (two or more layers of granulosa cells surrounding the oocyte). However, most of the ovarian follicles become atretic after initiation of growth and the developing stage. To avoid atresia of follicles, several methods were developed to rescue these follicles and then stimulate their activation and growth *in vitro* (Smitz and Cortvridnt, 2002); however, an optimal culture system for primordial follicles is still not available.

Minimal essential medium (MEM) has been used for culturing ovarian cortical tissue rich in primordial follicles (human: Wright *et al.*, 1999; bovine: Braw-Tal and Yossefi, 1997; caprine: Silva *et al*., 2004) as well as isolated secondary follicles (bovine: Figueiredo *et al*., 1994; murine: Zhao *et al*., 2001). Coconut water solution (CWS) is a natural solution, inexpensive, and rich in nutrients like proteins, sugars, vitamins, salt, neutral lipids, and substances that can induce cellular division (Santos *et al*., 1996; Laguna and Nunes, 1997). In addition, it contains electrolytes that can promote the survival and viability of female gametes (Martins *et al*., 2005). Indole-3-acetic acid (IAA), an important molecule present in coconut water, belongs to an auxin group and has a beneficial effect on the metabolism of goat sperm by increasing motility and fertility rates after preservation (Nunes and Combarnous, 1995). The presence of IAA and epidermal growth factor (EGF) in the culture medium of ovine cortical tissue has improved the viability of primordial follicles after *in vitro* culture (Andrade *et al*., 2005). According to Moon *et al*. (1994), the interaction between the animal EGF with IAA improves growth in various plants. Therefore, Silva *et al.* (2004) hypothesized that the IAA present in coconut water can bind to animal growth factors present in the ovarian tissue, and the complex can modulate the action of growth factors. Additionally, coconut water solution has been used for *in vitro* culture of goat primordial follicles (Silva *et al*., 2004; Martins *et al*., 2005) and preservation of ovaries in sheep (Andrade *et al*., 2002). However, the effectiveness of supplementing MEM with various proportions of CWS for promoting activation and viability of ovine primordial follicles after culturing of ovarian cortical tissue is unknown. The objective of this work was to investigate the effects of MEM, CWS, or MEM supplemented with 5, 10, 20, 50, 80, 90, or 95% CWS on viability, activation, and further growth of ovine primordial follicles cultured for 1, 3 or 5 days.

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Materials and Methods

Source and preparation of ovarian tissue

Ovaries (n = 10) from 5 adult (1 – 2 years old), mixed-breed sheep were obtained at a local slaughterhouse. Immediately after slaughter, adhering tissues were removed from the ovaries, which were subsequently washed once in 70% alcohol and twice in 0.9% saline solution. The material was transported in thermos flasks at 37 ºC to the laboratory within 1 hour and then submitted to the experimental procedure (as described below).

In vitro culture of sheep primordial follicles

At the laboratory, the ovaries were cut in half and the medulla, large antral follicles, and corpora lutea were removed. Following this, the ovarian cortex was divided in fragments approximately 3 x 3 mm (1 mm thick). One fragment was randomly taken and immediately fixed for histological examination (uncultured control – *in situ*), and another fragment was used for follicle isolation. Then, 30 fragments of ovarian cortex from each pair of ovaries were cultured *in vitro* individually in 20 four-well culture dishes (Biossystems, Brazil) containing 1 ml of MEM (Sigma, St Louis, MO, USA), CWS (50% of pure coconut water + 25% of water + 25% of sodium citrate solution), or MEM supplemented with 7 different concentrations of CWS (see Table 1). Another 3 fragments were cultured *in vitro* individually in MEM for 5 days and fixed for histology (cultured control – *in situ*). Culture media were supplemented with antibiotics (100 µg/ml of penicillin, 100 µg/ml of streptomycin), 0.25 µg/ml amphotericin B, 10% of fetal calf serum, insulin (5 µg/ml), transferrin (5.5 µg/ml), selenium (5 ng/ml; ITS), 0.23 mM pyruvate, 2 mM glutamine, and 2 mM hypoxanthine as described by Figueiredo *et al.* (1994). Unless indicated, the reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Fragments were cultured *in vitro* for 1, 3, or 5 days in an incubator at 39 °C and 5% CO_2. Culture medium was replaced every 48 hours.

Table 1. Composition of media used for *in vitro* culture of sheep preantral follicles.

Media	% MEM	% CWS
1	100	0
2	95	5
3	90	10
4	80	20
5	50	50
6	20	80
7	10	90
8	5	95
9	0	100

Follicular isolation

Sheep preantral follicles (primordial and developing) were isolated from ovarian fragments using a mechanical procedure described by Amorim *et al.* (2000). In brief, the ovarian cortex was cut at room temperature (25 °C) into small fragments with a tissue chopper at serial sections of 87.5 µm. The ovarian fragments were then placed in PBS supplemented with 0.1% (v/v) penicillin/streptomycin (Gibco, Paisley, UK) and subsequently pipetted 40 times with a Pasteur pipette to release the preantral follicles. The suspension was successively filtered through 500 and 100 µm nylon mesh filters. Preantral follicles, smaller than 100 µm in diameter, were collected under a stereomicroscope and submitted to viability analysis.

Analysis of follicular viability, activation and growth

At least 50 isolated follicles from each treatment were evaluated by adding 15 µl of 0.4% trypan blue (Sigma Chemicals, Poole, Dorset, UK) for every 300 µl of MEM and incubated for 1 minute (room temperature). After this, follicles were examined using an inverted microscope and then classified as nonviable when stained with trypan blue and viable if unstained. Nonviable follicles had oocytes and/or granulosa cells stained with trypan blue. The percentages of viable follicles were calculated for each experimental condition.

To evaluate follicular activation, percentages of primordial and developing follicles were calculated before culture (Day 0) and after 1, 3, or 5 days of culture in each treatment. Follicles were classified as primordial or developing. In addition, follicular diameter of intact follicles was measured before and after *in vitro* culture using an ocular micrometer.

Histology

To compare data from isolated follicles with those obtained by histology, either immediately after recovery (uncultured control – *in situ*) or after culture in MEM for 5 days (cultured control – *in situ*), fragments of ovarian tissue were fixed by immersion in buffered,

10% formaldehyde (pH 6.8-7.2) for 18 hours, dehydrated in a graded series of ethanol solutions, clarified with xylene, and embedded in paraffin wax. Thereafter, 5-µm sections of each fragment were cut and mounted on glass microscope slides. The slides were stained with periodic acid Schiff (PAS) and hematoxylin, and the percentage of normal primordial and developing follicles was determined.

Statistical analyses

Analysis of variance and Fisher PLSD tests (StatView for Windows) were used to compare the percentages of viable follicles as well as the percentages of primordial and developing follicles and follicular diameter among treatments. Values were considered statistically significant at $P < 0.05$.

Results

A total of 2367 follicles were examined to determine viability based on trypan blue staining. Morphologically normal (Fig. 1A) and degenerated follicles (Fig. 1B) in addition to viable (Fig. 2A) and nonviable (Fig. 2B and 2C) follicles are shown. Culture of ovarian tissue for 1, 3, or 5 days reduced ($P < 0.05$) the percentage of viable primordial and developing follicles when compared to noncultured cortical tissue, except for MEM with 20% CWS after 1 day of culture (Fig. 3). The increase in duration of culture period from 1 to 3 days significantly reduced the percentage of intact follicles, except for MEM with 50% CWS. In contrast, after *in vitro* culture in MEM with 10, 20, and 50% of CWS, a significant reduction in viable follicles was observed with the increase in duration of culture period from 3 to 5 days. After 5 days of culture, MEM alone or added by 5% CWS had a significantly higher percentage of viable follicles when compared to all other media. Evaluation of histological sections from uncultured (Day 0) and cultured tissue for 5 days in MEM showed 89.9 $\pm$ 4.2% and 72.7% $\pm$ 7.0% normal follicles, respectively. These results were not significantly different from those obtained with isolated follicles before and after culture in the same conditions. After culture of ovarian tissue in MEM with 80, 90, 95%, and pure CWS, a similar percentage of intact follicles was observed (Fig. 3).

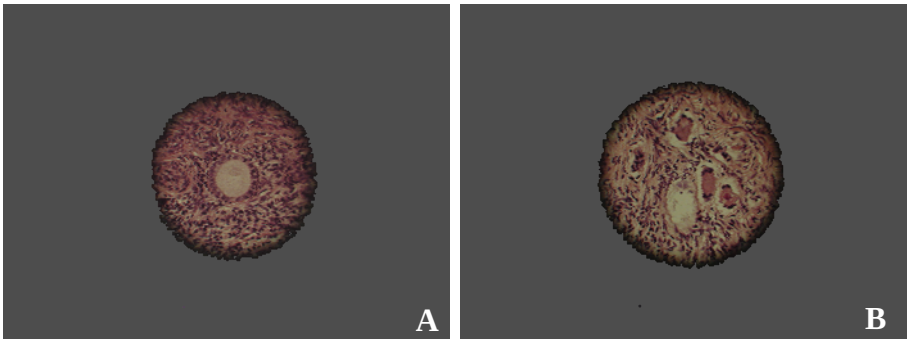

Figure 1. Histological sections of morphologically normal primary (A) and degenerated primordial (B) ovine follicles after PAS-hematoxylin staining (400x).

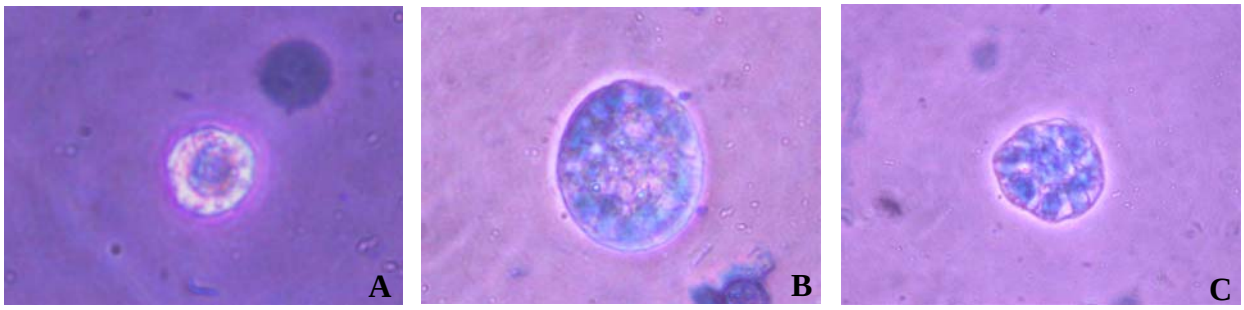

Figure 2. Viable (A) and nonviable (B, C) ovine follicles after trypan blue staining (600x).

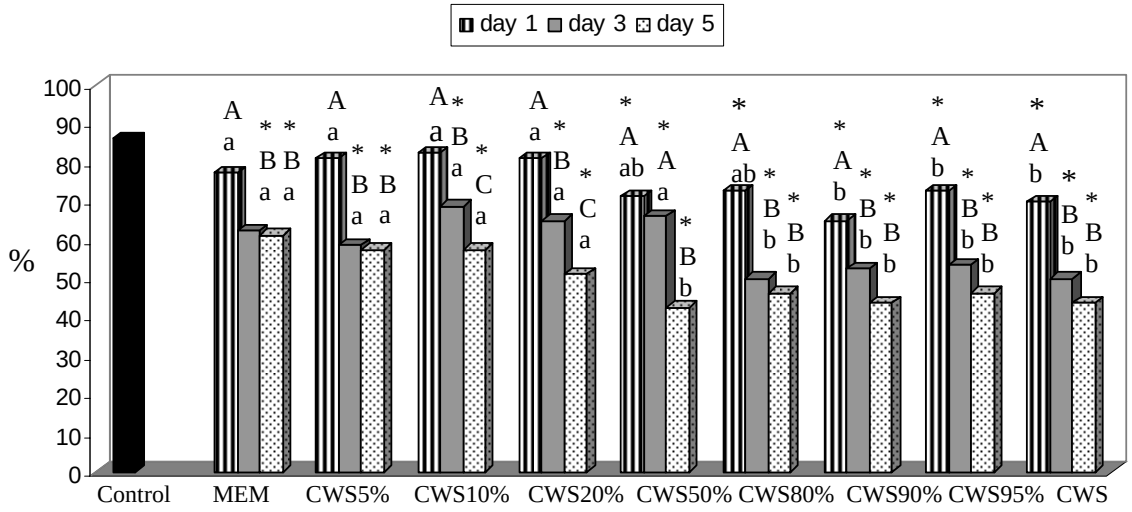

Figure 3. Percentages of viable ovine follicles before and after *in vitro* culture for 1, 3, or 5 days .
*Differs significantly from **noncultured** tissue (control);
A,B,C – Differs among days of culture within each medium (P < 0.05);
a,b – Differs among media within the same days of culture (P < 0.05).

The percentage of viable primordial and developing follicles in noncultured ovarian cortex is showed in Table 2. After 1 day of culture, the percentage of viable primordial or developing follicles did not differ significantly from controls for all treatments. On the other hand, after 5 days of culture, the percentage of viable primordial follicles was reduced (P < 0.05) when MEM with up to 10% CWS was used and resulted in a concomitant increase (P < 0.05) in the percentage of developing follicles compared to noncultured tissue. After evaluating histological sections from uncultured (Day 0) and cultured tissue for 5 days in MEM, a significant reduction in the percentage of primordial follicles (from 63.0 ± 5.4% to 20.6 ± 4.6%, respectively) and increase of development follicles (from 37.0 ± 5.4% to 79.4 ± 4.6%, respectively) was observed. No significant differences were observed between data obtained from histological evaluation and isolated follicles.

Table 3 shows follicle diameter in noncultured and *in vitro* cultured ovarian cortex (1, 3, or 5 days). After 5 days of culture using MEM only or MEM with 10% CWS, a significant increase in follicle diameter was observed when compared to follicles in noncultured tissue or in tissue cultured for 1 or 3 days.

Table 2. Percentage (mean ± SD) of viable sheep primordial and developing follicles before (Day 0) and after *in vitro* culture for 1, 3, or 5 days.

	Control					
	Primordial 91.0 ± 9.6			Developing 9.0 ± 9.6		
	Day 1		Day 3		Day 5	
Medium	Primordial	Developing	Primordial	Developing	Primordial	Developing
MEM	70.0 ± 14.6^{aA}	30.0 ± 14.6^{aA}	71.0 ± 15.1^{aA}	28.4 ± 14.8^{aA}	$43.0 \pm 37.2^{bB*}$	$37.0 \pm 35.1^{bB*}$
CWS 5%	77.0 ± 17.2^{aA}	23.0 ± 17.2^{aA}	68.0 ± 9.1^{aA}	32.0 ± 9.1^{aA}	$67.4 \pm 12.1^{bB*}$	$32.4 \pm 11.9^{bB*}$
CWS 10%	79.0 ± 10.8^{aA}	21.0 ± 10.8^{aA}	72.0 ± 9.1^{aA}	28.0 ± 9.1^{aA}	$60.0 \pm 21.5^{bB*}$	$40.0 \pm 21.5^{bB*}$
CWS 20%	78.0 ± 11.5^{aA}	22.0 ± 11.5^{aA}	79.8 ± 16.3^{aA}	20.2 ± 16.3^{aA}	75.0 ± 13.7^{aA}	25.0 ± 13.7^{aA}
CWS 50%	82.0 ± 5.7^{aA}	18.0 ± 5.7^{aA}	79.0 ± 12.9^{aA}	21.0 ± 12.9^{aA}	68.0 ± 13.9^{aA}	32.0 ± 13.9^{aA}
CWS 80%	76.0 ± 12.9^{aA}	24.0 ± 12.9^{aA}	75.4 ± 16.2^{aA}	24.6 ± 16.2^{aA}	71.8 ± 14.7^{aA}	28.1 ± 14.7^{aA}
CWS 90%	85.0 ± 11.2^{a}	15.0 ± 11.2^{aA}	86.0 ± 8.9^{aA}	14.0 ± 8.9^{aA}	65.0 ± 21.8^{aA}	35.0 ± 21.8^{aA}
CWS 95%	74.0 ± 8.9^{aA}	26.0 ± 8.9^{aA}	74.0 ± 10.8^{aA}	26.0 ± 10.8^{aA}	66.0 ± 10.8^{aA}	34.0 ± 10.8^{aA}
CWS	79.0 ± 21^{aA}	21.0 ± 21^{aA}	79.0 ± 15.6^{aA}	21.0 ± 15.6^{aA}	78.3 ± 5.2^{aA}	21.7 ± 5.2^{aA}

*Differs significantly from noncultured tissue (control);
A,B – Differs among days of culture within each medium (P < 0.05).
a,b – Differs among media within the same days culture (P < 0.05).

Table 3. Follicle diameter (media ± SD) of ovine primordial and developing follicles before (day 0) and after in vitro culture for 1, 3 or 5 days.

	Control					
	Primordial			Developing		
	20.5±2.0			40.8±3.7		
	Day 1		Day 3		Day 5	
Medium	Primordial	Developing	Primordial	Developing	Primordial	Developing
MEM	24.8 ± 0.7^{aA}	41.8 ± 5.6^{aA}	25.0 ± 0.9^{aA}	38.6 ± 5.3^{aA}	$27.7 \pm 2.0^{bB*}$	$41.3 \pm 2.6^{bB*}$
CWS 5%	23.8 ± 2.0^{aA}	37.6 ± 3.4^{aA}	24.3 ± 0.8^{aA}	37.7 ± 3.8^{aA}	23.6 ± 1.2^{aA}	37.3 ± 3.7^{aA}
CWS 10%	24.3 ± 1.9^{aA}	40.7 ± 2.8^{aA}	24.6 ± 1.7^{aA}	40.8 ± 4.3^{aA}	$27.0 \pm 2.1^{bB*}$	$40.9 \pm 3.0^{bB*}$
CWS 20%	23.3 ± 1.6^{aA}	37.1 ± 2.5^{aA}	24.1 ± 1.9^{aA}	40.4 ± 7.9^{aA}	25.0 ± 1.0^{aA}	37.7 ± 6.9^{aA}
CWS 50%	24.5 ± 3.2^{aA}	38.3 ± 4.8^{aA}	24.0 ± 2.7^{aA}	37.9 ± 4.8^{aA}	24.8 ± 2.2^{aA}	41.1 ± 5.3^{aA}
CWS 80%	24.6 ± 2.7^{aA}	39.6 ± 2.8^{aA}	24.4 ± 2.5^{aA}	39.9 ± 4.9^{aA}	25.1 ± 2.7^{aA}	37.1 ± 4.1^{aA}
CWS 90%	23.1 ± 4.3^{aA}	41.2 ± 6.0^{aA}	24.1 ± 2.11^{aA}	41.1 ± 2.6^{aA}	24.7 ± 2.0^{aA}	38.4 ± 2.8^{aA}
CWS 95%	23.5 ± 2.4^{aA}	39.0 ± 4.7^{aA}	23.8 ± 2.2^{aA}	39.4 ± 3.0^{aA}	24.8 ± 1.3^{aA}	39.4 ± 1.1^{aA}
CWS	25.4 ± 3.7^{aA}	38.3 ± 3.7^{aA}	24.8 ± 3.1^{aA}	39.4 ± 4.1^{aA}	23.5 ± 1.3^{aA}	38.4 ± 2.3^{aA}

*Differs significantly from noncultured tissue (control);
A,B – Differs significantly among days of culture within each medium ($P < 0.05$).
a,b – Differs significantly among media within the same days of culture ($P < 0.05$).

Discussion

This study showed that ovine primordial follicles can be activated after *in vitro* culture in MEM alone or MEM supplemented with 5 or 10% CWS, but the percentages of developing and viable follicles decreased with increasing proportions of CWS mixed with MEM. After culture, the percentages of primordial and developing follicles were significantly reduced when compared to control values. Similar results were obtained in studies that used bovine (Wandji *et al.* 1996; Braw-Tal and Yossefi, 1997) and baboon (Fortune *et al*., 1998) ovarian fragments; the number of primordial follicles was dramatically reduced after 2 days of culture with a concomitant increase in the number of developing follicles.

In this study, after 5 days of culture, the supplementation of MEM with 5 and 10% CWS kept the percentage of developing follicles similar to that of tissue cultured in MEM whereas the use of 20% CWS or higher as well as pure CWS decreased the percentage of developing follicles. In a report from our group (Martins *et al*., 2005), the addition of 20% or more of CWS to MEM also reduced the number of developing goat follicles. The percentages of degenerated follicles observed by these authors for all treatments were similar to those observed in the current study and significantly increased with use of pure CWS. Coconut water solution has been used successfully for *in vitro* preservation of ovine ovarian follicles (Andrade *et al*., 2002), dog semen (Cardoso *et al*., 2002), and culture of ovine primordial follicles (Silva *et al*., 2004). However, we suggest that CWS negatively influenced follicular development depending on the proportion used.

There are many methods to assess the success of follicular cell culture like histological analysis, transmission electronic microscopy, and vital staining with trypan blue. Trypan blue exclusion is a test used to detect damage to the plasma membrane integrity and thus to asses cell viability (Isayeva *et al.*, 2004; Santos *et al*., 2007). Amorim *et al.* (2003) showed that to evaluate the survival of ovarian follicles, staining with trypan blue and histological analysis yielded similar results. Furthermore, Ostrowska *et al.* (2000) demonstrated that the use of trypan blue to assess the viability of cryopreserved isolated human hepatocytes is as efficient as flow cytometry analysis. In this study, similar percentages of developing follicles were observed after evaluating histological sections of cultured tissue and trypan-blue stained follicles.

The percentages of viable follicles in noncultured tissue were similar to those described previously for sheep after histological analysis (Andrade *et al*., 2002). After 1 day of culture, MEM was the only medium that kept the percentage of normal follicles at the level of uncultured control tissue. Gupta *et al.* (2002) reported that MEM can be successfully used for *in vitro* culture of buffalo follicles for a long period (40 days) but requires supplementation to support follicular survival and growth. Wright *et al.* (1999) also described that MEM is more effective in promoting human primordial follicle growth than Waymouth´s medium and Earle's balanced salt solution with 10% human serum. The supplements added to MEM contributed to the survival of cultured, early-stage follicles from goats (Silva *et al*., 2004), cats (Jewgenow, 1998), cows (Figueiredo *et al.*, 1994), and humans (Wright *et al.*, 1999). After 5 days of culture, the results showed that CWS reduces follicle viability when added at increasing proportions. Silva *et al.* (2004) also demonstrated a

reduction in the percentage of normal goat follicles after culture in medium containing CWS. The ineffectiveness of CWS in maintaining follicle viability could be due to variability of coconut water composition because of the area of origin and age of the plant. Thus, CWS is not likely to replace MEM or other supplements like serum.

In summary, this study showed that sheep primordial follicles are activated *in vitro* in MEM, CWS, or mixed solutions composed of MEM in different proportions of CWS. Culture of cortical tissue in MEM with up 10% CWS kept the levels of primordial follicle activation similar to MEM. It was concluded that supplementation of MEM with different proportions of CWS does not improve primordial follicle survival, activation, or further growth.

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