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Effects of α-MEM and TCM-199 culture media and epidermal growth factor on survival and growth of goat and sheep preantral follicles cultured *in vitro*

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Abstract

The aim of this study was to evaluate the effect of culture media (Alpha Minimum Essential Medium, α-MEM; and Tissue Culture Medium-199, TCM-199) in the absence or presence of Epidermal Growth Factor (EGF) on an *in vitro* culture of goat and sheep preantral follicles enclosed in ovarian tissue. The fragments of ovarian cortex from both species were immediately analyzed after collection (non-cultured control group) or cultured for 1 or 7 days in α-MEM$^+$ or TCM-199$^+$ in the absence or presence of EGF (10 ng/ml). Before and after the culture, the fragments of ovarian cortex were analyzed by classical histology and fluorescence microscopy. After 1 day of culture, all treatments decreased the percentage of morphologically normal follicles when compared to non-cultured control in both species ($P < 0.05$). In fluorescence microscopy, viable sheep follicles were observed to decrease in all treatments after 7 days of culture when compared to non-cultured controls. However, in goats, the culture with TCM-199$^+$ maintained follicle viability after 7 days of culture, similar to fresh tissue ($P > 0.05$). Regarding follicle activation, an increase in the percentage of growing follicles was observed in all treatments after 7 days of culture when compared to the control group in both species. However, in sheep, after 7 days, only the treatments α-MEM$^+$/EGF and TCM-199$^+$ showed larger ($P < 0.05$) oocytes than the control group. In conclusion, the TCM-199$^+$ preserved goat preantral follicle viability after *in vitro* culture. Furthermore, the media α-MEM$^+$/EGF and TCM-199$^+$ increased the oocyte diameter after 7 days of culture in sheep. Therefore, it is recommended to use TCM-199$^+$ in the culture of preantral follicles in both species.

Keywords: goat, preantral follicle, sheep, TCM-199, α-MEM.

Introduction

Several studies using *in vitro* culture of preantral follicles have been performed to obtain larger numbers of mature oocytes. However, to obtain a greater number of mature oocytes after *in vitro* culture, it is necessary to know and investigate the role of different substances added to the culture medium in the activation and growth processes of early preantral follicles in each species.

Among commonly used culture media are the Minimum Essential Medium (MEM; Silva *et al.*, 2004; Chaves *et al.*, 2008), the Alpha Minimum Essential Medium or the modified MEM (α-MEM; Chaves *et al.*, 2010; Faustino *et al.*, 2011), and the Tissue Culture Medium 199 (TCM-199; Javed *et al.*, 2010; Rossetto *et al.*, 2012). These media (α-MEM and TCM-199) have been used alone or added with other substances in the *in vitro* culture of goat and sheep preantral follicles, and were able to ensure the survival and viability of the follicles and to promote early follicular development (Hemamalini *et al.*, 2003; Chaves *et al.*, 2011; Lima *et al.*, 2013). In addition, growth factors such as EGF promoted *in vitro* survival maintenance, viability, and development of preantral follicles enclosed in goat and sheep ovarian tissues (Andrade *et al.*, 2005; Celestino *et al.*, 2009).

Although there are studies using various types of culture media in goats and sheep, there are no studies comparing the relative efficiency of α-MEM and TCM-199 media in the presence or absence of EGF on preantral folliculogenesis in these species, under the same experimental conditions. Thus, the aim of this study was to evaluate the efficiency of culture media α-MEM and TCM-199 in the presence or absence of EGF on survival, growth, and viability of goat and sheep preantral follicles enclosed in ovarian tissues.

Materials and Methods

Chemicals

Unless stated otherwise, the culture media and other chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO).

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Source of ovaries, groups, and culture

Ovaries were collected at a local slaughterhouse from mixed-breed goats (n = 5) and sheep (n = 5). In the laboratory, the cortex from each pair of ovaries was removed and cut into 9 fragments (9 mm^3). The fragments were placed in MEM with HEPES and then one slice of tissue was immediately fixed for histological analysis (non-cultured control: day 0). The remaining slices of ovarian cortex were cultured individually for 1 or 7 days in 1 ml of a culture medium in 24-well culture dishes at 39°C, in an atmosphere of 5% CO_2 in air. The basic culture media, referred to as α-MEM^+ or TCM-199^+, consisted of α-MEM or TCM-199 supplemented with 5.5 µg/ml transferrin, 5 ng/ml selenium, 2 mM glutamine, 2 mM hypoxantine, 1.25 mg/ml bovine serum albumin, and antibiotics (100 µg/ml penicillin and 100 µg/ml streptomycin) for both species. The insulin concentrations were different between species: 10 ng/ml for goats (Chaves *et al.*, 2011) and 10 µg/ml for sheep (Lima *et al.*, 2013). The medium was supplemented or not with EGF (10 ng/ml). Therefore, the groups were named: α-MEM^+, α-MEM^+/EGF, TCM^+, and TCM^+/EGF. The EGF concentration was chosen based on a previous study (Celestino *et al.*, 2009). Every 2 days, all the culture medium was replaced.

Histological processing

After fixation, non-cultured control and cultured tissues were dehydrated in ethanol, clarified with xylene, and embedded in paraffin wax. Serial sections (7 µm) of tissue were cut and every fifth section was mounted on glass slides and stained with periodic acid Schiff (PAS) - hematoxylin. Preantral follicles were classified according to developmental stages (Silva *et al.*, 2004) as: primordial (one layer of flattened granulosa cells around the oocyte); primary (one layer of cuboidal granulosa cells); and secondary (two or more layers of cuboidal granulosa cells around the oocyte and no sign of antrum formation). Follicles were classified individually as histologically normal when an intact oocyte was present and surrounded by granulosa cells that were well organized in one or more layers and had no pyknotic nuclei. Atretic follicles were defined as those with a retracted oocyte, pyknotic nucleus, and/or disorganized granulosa cells detached from the basement membrane (Gutierrez *et al.*, 2000). Follicular viability was assessed by epifluorescence microscopy (Fig. 1) using a marker for live (calcein-AM) or dead (ethidium homodimer-1) cells, as previously described (Lima *et al.*, 2013).

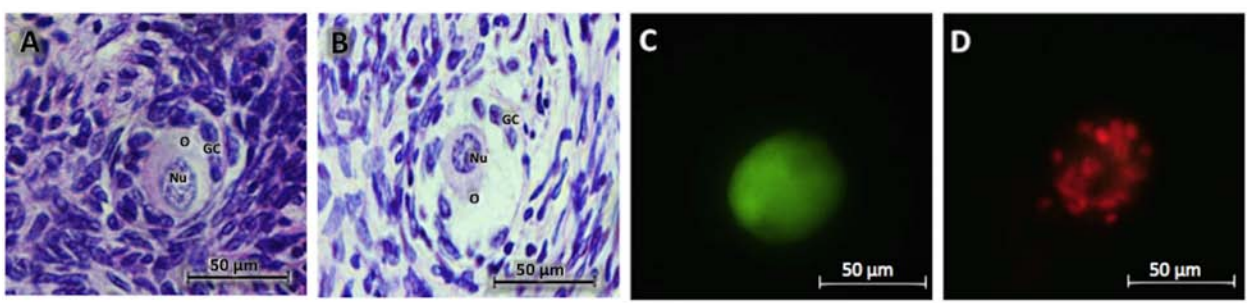

Figure 1. Photomicrographs of preantral follicles analyzed by histology and fluorescent microscopy. Histological sections after staining with periodic acid Schiff-hematoxylin, showing (A) non-cultured control normal preantral follicle and (B) degenerated follicle after culture in medium α-MEM^+ for 7 days. Note the retracted oocyte with a pyknotic nucleus (B). GC: granulosa cell; O: Oocyte; Nu: oocyte nucleus. Assessment of the viability of preantral follicles using fluorescent probes after culture for 7 days. (C) Isolated preantral follicle after *in vitro* culture in medium TCM-199^+ marked in green by calcein-AM, and (D) non-cultured control degenerated preantral follicle marked in red by ethidium homodimer-1.

Statistical analyses

Follicular viability evaluated by fluorescent markers (discrete variable) was analyzed using Chi-square test and results were expressed as percentages. Continuous variables such as follicular survival and activation were analyzed using PROC MIXED of SAS, 2002, including a repeated statement to account for autocorrelation between sequential measurements. Comparisons among culture media were further analyzed by the LSD test. A probability of $P < 0.05$ indicated a significant difference and results were expressed as mean ± S.D.

Results

For both species, irrespective of day of culture, there was no difference among the treatments in the percentage of morphologically normal and developing follicles or in the follicular and oocyte diameters. From day 1 onwards of *in vitro* culture, all treatments reduced ($P < 0.05$) the percentage of morphologically normal follicles when compared to the non-cultured control group in both species (Table 1). Only in goats was there a reduction ($P < 0.05$) in the percentage of morphologically normal follicles in all treatments with the progression of the culture period.

Table 1. Mean (± SD) percentage of morphologically normal sheep and goat preantral follicles in the fresh control (non-cultured) group and after 1 or 7 days of culture in medium α-MEM$^+$ and TCM-199$^+$ in the absence or presence of EGF.

	Normal follicles (%)			
Treatments	Sheep		Goat	
Control (Day 0)	84.0 ± 6.4		92.7 ± 1.5	
	Day 1	Day 7	Day 1	Day 7
α-MEM$^+$	71.3 ± 3.8^{*,A}	70.0 ± 9.7^{*,A}	86.0 ± 4.4^{*,A}	68.7 ± 8.7^{*,B}
α-MEM$^+$/EGF	66.0 ± 4.4^{*,A}	66.7 ± 5.3^{*,A}	82.7 ± 7.6^{*,A}	71.3 ± 6.9^{*,B}
TCM-199$^+$	67.3 ± 9.4^{*,A}	68.7 ± 10.7^{*,A}	77.5 ± 7.4^{*,A}	72.0 ± 6.9^{*,B}
TCM-199$^+$/EGF	70.7 ± 7.2^{*,A}	63.3 ± 6.7^{*,A}	86.0 ± 4.9^{*,A}	76.7 ± 2.4^{*,B}

*Value differs ($P < 0.05$) from the non-cultured control within the same species. ^{A,B}Between columns (days of culture) within the same species, treatments without a common superscript differed ($P < 0.05$). Within each day of culture, no difference was observed among treatments for any species.

Because EGF addition did not affect ($P > 0.05$) the percentage of morphologically normal follicles, only treatments in the absence of EGF were analyzed by fluorescence microscopy. In quantitative analysis by fluorescence microscopy, we observed that both culture media decreased the percentage of viable follicles in the end of culture when compared to the control group in sheep (Fig. 2). However, in goats, only the treatment with α-MEM$^+$ decreased ($P < 0.05$) the percentage of viable follicles when compared to the control. Furthermore, TCM-199$^+$ maintained follicular viability after 7 days of culture when compared with the control group.

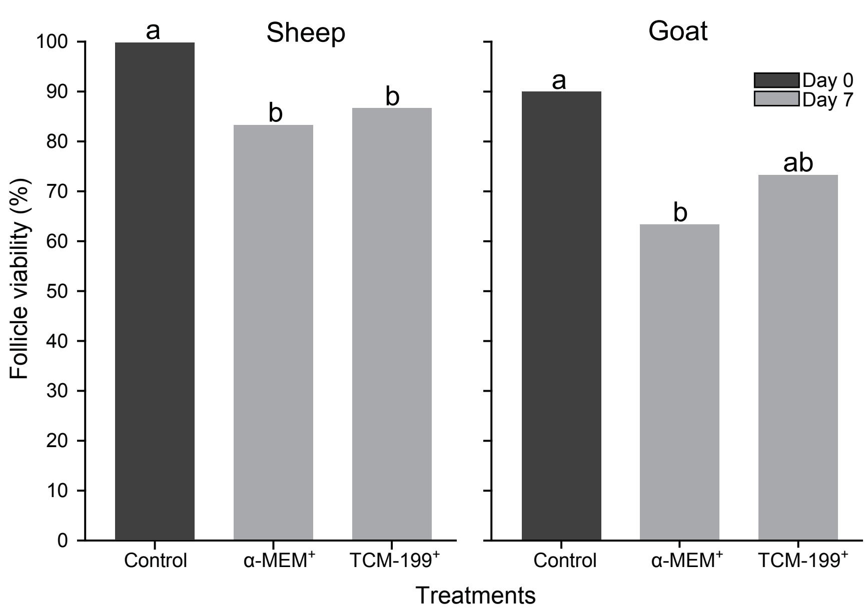

Figure 2. Percentages of viable sheep and goat preantral follicles in the non-cultured control group and after 7 days of culture in medium α-MEM$^+$ and TCM-199$^+$. ^{a,b}Values without a common superscript differed ($P < 0.05$) among treatments.

After 7 days of culture, the proportion of primordial follicles was reduced ($P < 0.05$) as a result of an increase in the proportion of developing follicles (transition, primary, and secondary). In all treatments (Table 2), it is noteworthy that for goats, a precocious follicular development was observed in both culture media used, since there was an increase ($P < 0.05$) in the percentage of developing follicles already on day 1 of culture when compared to the non-cultured control group.

Follicular and oocyte diameters in non-cultured control (fresh tissue) and after 1 and 7 days of culture in both species are shown (Table 3). In goats, follicular and oocyte diameters were similar ($P > 0.05$) among control and other treatments. However, in sheep, after 1 day of culture an increase ($P < 0.05$) in oocyte diameter was observed in all treatments when compared to the control group. After 7 days, only α-MEM$^+$ in presence of EGF and the TCM-199$^+$ alone produced oocytes larger ($P < 0.05$) than in the control group. Moreover, with the progression of the culture period, there was a reduction ($P < 0.05$) of oocyte diameter in treatment TCM-199$^+$ in presence of EGF.

Table 2. Mean (± SD) percentage of developing sheep and goat preantral follicles in the fresh control (non-cultured) group and after 1 or 7 days of culture in medium α-MEM^{+} and TCM-199^{+} in the absence or presence of EGF.

	Developing follicles (%)			
Treatments	Sheep		Goat	
Control (Day 0)	27.1 ± 5.1		23.0 ± 6.1	
	Day 1	Day 7	Day 1	Day 7
α-MEM^{+}	19.4 ± 5.0^{A}	$76.4 \pm 14.9^{*,B}$	$53.6 \pm 6.9^{*,A}$	$78.7 \pm 9.0^{*,B}$
α-MEM^{+}/EGF	18.1 ± 10.2^{A}	$82.0 \pm 7.2^{*,B}$	$62.5 \pm 10.2^{*,A}$	$82.8 \pm 8.0^{*,B}$
TCM-199^{+}	21.7 ± 7.8^{A}	$78.8 \pm 3.5^{*,B}$	$59.0 \pm 8.4^{*,A}$	$85.3 \pm 4.5^{*,B}$
TCM-199^{+}/EGF	16.4 ± 11.1^{A}	$69.4 \pm 14.9^{*,B}$	$59.4 \pm 10.8^{*,A}$	$85.3 \pm 3.6^{*,B}$

*Value differs ($P < 0.05$) from the non-cultured control within the same species. ^{A,B}Between columns (days of culture) within the same species, treatments without a common superscript differed ($P < 0.05$). Within each day of culture, no difference was observed among treatments for any species.

Table 3. Mean (± SD) follicular and oocyte diameter of sheep and goat preantral follicles in the fresh control (non-cultured) group and after 1 or 7 days of culture in medium α-MEM^{+} and TCM-199^{+} in the absence or presence of EGF.

	Sheep				Goat			
Treatments	Follicular diameter (µm)		Oocyte diameter (µm)		Follicular diameter (µm)		Oocyte diameter (µm)	
Control (Day 0)	32.0 ± 8.5		1 4.2 ± 2.6		32.7 ± 5.2		23.5 ± 4.9	
	Day 1	Day 7	Day 1	Day 7	Day 1	Day 7	Day 1	Day 7
α-MEM^{+}	28.4 ± 4.3^{A}	30.2 ± 8.8^{A}	$19.0 \pm 3.0^{*,A}$	17.4 ± 5.3^{A}	31.5 ± 3.8^{A}	29.8 ± 5.4^{A}	21.7 ± 2.9^{A}	19.7 ± 3.0^{A}
α-MEM^{+}/EGF	29.9 ± 4.0^{A}	29.4 ± 6.6^{A}	$20.2 \pm 3.5^{*,A}$	$18.3 \pm 4.4^{*,A}$	32.7 ± 4.7^{A}	32.2 ± 5.6^{A}	22.0 ± 4.1^{A}	20.2 ± 4.9^{A}
TCM-199^{+}	28.1 ± 3.7^{A}	32.5 ± 8.1^{A}	$19.6 \pm 1.4^{*,A}$	$18.5 \pm 4.9^{*,A}$	29.3 ± 3.3^{A}	31.4 ± 8.8^{A}	20.6 ± 3.3^{A}	19.8 ± 5.4^{A}
TCM-199^{+}/EGF	30.5 ± 4.0^{A}	25.8 ± 6.0^{A}	$20.6 \pm 2.8^{*,A}$	15.9 ± 5.2^{B}	31.7 ± 4.7^{A}	31.5 ± 7.6^{A}	21.6 ± 3.2^{A}	20.3 ± 5.5^{A}

*Value differs ($P < 0.05$) from the non-cultured control within the same species. ^{A,B}Between columns (days of culture) within the same end point and species, treatments without a common superscript differed ($P < 0.05$). Within each day of culture, no difference was observed among treatments for any end point and species.

Discussion

The present study evaluated the effect of culture media α-MEM^{+} and TCM-199^{+} in presence or absence of EGF in the morphology, viability, and growth of goat and sheep preantral follicles. In both species and culture media there was a reduction in percentage of morphologically normal follicles after 7 days of culture when compared to non-cultured control. Regarding follicle activation, i.e. the primordial to primary follicle transition, after 7 days of culture, we observed an increase in the percentage of developing follicles in all treatments in goats and sheep compared to the control. This activation result is probably due to the rich composition of the used media (α-MEM and TCM-199), which are composed of several nutrients, such as amino acids, antioxidants, inorganic salts, and glucose, that may stimulate follicular activation (Javed *et al.*, 2010; Haag *et al.*, 2013). Amino acids provide energy sources and precursor protein synthesis (Fujihara *et al.*, 2012), and antioxidants responsible for protecting cells against reactive oxygen species (ROS; Rossetto *et al.*, 2009).

In the present study, the addition of EGF in the culture media did not have an effect on the survival, activation, and growth of preantral follicles enclosed in fragments of ovarian tissue. However, in goats (Celestino *et al.*, 2009) and sheep (Andrade *et al.*, 2005) there was a beneficial effect of the addition of EGF on survival and follicular development after culture of preantral follicles enclosed in ovarian tissue. The divergence in results might have been related to the differences between the used culture media, since these studies used MEM, which is a simple medium with inorganic salts and glucose. The simple composition of this medium was not sufficient for follicular development; thus the effect of EGF was more evident. In addition, other differences in chemicals used between our study and the studies of Celestino *et al.* (2009) and Andrade *et al.* (2005) might have promoted distinct results. Because previous studies used human recombinant EGF (rEGF), the reason for one of the differences observed in the current study might have been the source of the EGF utilized. In our study, we used the EGF from mouse submaxillary glands, which has reported positive effects in goat secondary follicle growth (Silva *et al.*, 2013). Furthermore, a study with recombinant follicle stimulating hormone (rFSH) has shown that different sources of substances might have an influence on the *in vitro* culture of cells (Magalhães *et al.*, 2009), which may have occurred in this study in regard to EGF.

Evaluation of follicular diameter showed no difference between days of culture in both species and culture media in presence or absence of EGF. However, in sheep, we observed an increase in the oocyte diameter after 7 days of culture in α-MEM^{+} medium supplemented with EGF and in TCM-199^{+} without the addition of EGF. This result clearly shows that, unlike α-MEM$^+$, when modified TCM-199$^+$ was used in the culture of ovine preantral follicles, no addition of growth factors was necessary for promoting oocyte growth. Differences in the medium composition (compound type and/or concentration) between the aforementioned media may explain this effect. For example, the content of some amino acids (arginine, glutamine, leucine, and tyrosine) is higher in TCM-199 when compared with α-MEM. In this regard, amino acids have been reported to improve cell viability in embryos (Gardner and Lane 2003), potentially by reducing oxidative stress mechanisms (Liu and Foote, 1995; Gardner, 2008). Furthermore, in ewe the administration of amino acids promoted an increase in ovulation rate (Downing *et al.*, 1995). Therefore, in this study the variable(s) which counterbalanced the additional effect of EGF remains to be investigated.

In conclusion, the medium TCM-199$^+$ preserved goat preantral follicle viability after *in vitro* culture. However, the media α-MEM$^+$/EGF and TCM-199$^+$ increased the oocyte diameter after 7 days of culture in ovine. Therefore, it is recommended to use TCM-199$^+$ in the culture of preantral follicles in both species.

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