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Effect of medium composition on the *in vitro* culture of bovine pre-antral follicles: morphology and viability do not guarantee functionality

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Summary

This study investigated the effect of three different culture media (α minimum essential medium (α-MEM), McCoy or TCM199 during the *in vitro* culture (IVC) of bovine isolated pre-antral follicles. Pre-antral follicles greater than 150 μm in size were isolated and cultured for 0 (control), 8 or 16 days in one of the abovementioned culture media. Follicles were evaluated for survival, growth and antrum formation at days 8 and 16. The results showed that TCM199 was the most suitable medium to preserve follicular viability and ultrastructure, resulting in the highest rates of antrum formation. In conclusion, TCM199 promotes the *in vitro* development of isolated pre-antral follicles without hampering follicular functionality by sustaining *in vitro* growth and antrum formation.

Keywords: Antrum, IVC, Pre-antral ovarian follicle, Ultrastructure, Viability

Introduction

In vitro culture (IVC) of pre-antral follicles emerges as an alternative to optimize the use of female gametes. Except for one report of live birth after complete *in vitro* development of murine primordial follicles (O'Brien *et al.*, 2003), such a procedure in large mammals remains a challenge. Success is restricted to antrum formation in bovine (McLaughlin *et al.*, 2010), and embryo production in bubaline (Gupta *et al.*, 2008), ovine (Arunakumari *et al.*, 2010) and caprine (Saraiva *et al.*, 2010) species after IVC of growing pre-antral follicles. The first step to develop a culture system is to supplement culture medium by adding hormones and growth factors. Although culture media are basically composed of vitamins, amino acids and salts (Figueiredo *et al.*, 2008), variety and concentrations of additives differ, which may affect follicular IVC and may explain the non-compliance of results obtained by different research groups, even though the medium was supplemented with the same hormones and growth factors. The most used media for IVC of pre-antral follicles are α-MEM (Saraiva *et al.*, 2010), McCoy (McLaughlin *et al.*, 2010) and TCM-199 (Arunakumari *et al.*, 2010). Differences among cell culture media may be neglected due to the methods commonly used to evaluate follicular quality, e.g. morphology and viability, which may not guarantee maintenance of follicular functionality during IVC. For example, *in vitro* growth and antrum formation may be used as parameters to

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evaluate follicular functioning. Therefore our aim was to evaluate the effect of three different culture media (α-MEM, McCoy and TCM-199) on the ultrastructure, viability, growth and antrum formation of isolated bovine pre-antral follicles.

Material and methods

All chemicals used were purchased from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO, USA). Ovaries from 12 adult mixed-breed cows were attained from a local slaughterhouse. Immediately after slaughter, the ovaries were washed once in 70% alcohol and minimum essential medium (MEM) supplemented with HEPES and antibiotics (100 mg/ml penicillin and 100 mg/ml streptomycin). Each ovarian pair was submitted to a microdissection procedure (Silva *et al.*, 2010) to recover pre-antral follicles >150 μm. Pre-antral follicles ($n = 119$) containing a visible oocyte surrounded by granulosa cells and with intact basement membrane were selected for IVC. For this technique, follicles were individually cultured in 96-well plates containing 150 μl of one of the tested culture medium in a humidified atmosphere of 5% CO_2 at 38.5 °C for 16 days. Tested culture media were α-MEM, McCoy and TCM199. All culture media were supplemented with 20 mM HEPES, 1% bovine serum albumin (BSA), 3 mM glutamine, 2.5 μg/ml transferrin, 4 ng/ml selenium, 50 μg/ml ascorbic acid, 10 ng/ml insulin, 100 ng/ml FSH 100 ng/ml, 100 ng/ml activin, 0.1 mg/ml penicillin and 0.1 mg/ml streptomycin as described by McLaughlin *et al.* (2010). Every 4 days, half of the culture medium was refreshed and follicles were evaluated at days 0, 8 and 16. Follicular viability was assessed by epifluorescence microscopy using a marker for live (calcein-AM) or dead (ethidium homodimer-1) cells as previously described (Santos *et al.*, 2006). Ultrastructural analysis followed the parameters applied by Nottola *et al.* (2008) with moderate modifications. Qualitative assessment included shape and dimensions of the nucleus, dispersion of the nucleus chromatin structure, presence of an intact oolemma, quality of the organelles, cytoplasmic vacuolization, and preserved contact between oocyte and surrounding granulosa cells (GC). Data were evaluated by using an analysis of variance (ANOVA) and Kruskal–Wallis test and $p < 0.05$ was considered statistically significant.

Results

A summary of the results is presented in Fig. 1. Although after 16 days of IVC the rates of viable follicles were lower when McCoy medium was used in comparison with α-MEM and TCM199, follicular viability was maintained above 70%. The use of TCM199 resulted in the highest percentages of viable follicles (Fig. 1*A*). Viable follicles (Fig. 1*B*,*C*) were positively stained with calcein-AM, while non-viable follicles were stained with ethidium homodimer (Fig. 1*D*,*E*). At the ultrastructural level, pre-antral follicles cultured in the presence of α-MEM and TCM199 presented integer cytoplasmic membrane, normal and uniformly distributed organelles, especially mitochondria and endoplasmic reticulum, and intact oocyte nucleus (Fig. 1*F*,*G*). However, when McCoy medium was used, follicles exhibited signals of vacuolization, multivesicular bodies in the ooplasm, GCs detached from the oocyte and disrupted basal membrane (Fig. 1*H*,*I*). Increase in follicular diameter after 8 days of IVC was significantly higher when follicles were cultured in TCM199 than in α-MEM and McCoy. Furthermore, progressive diameter increase from 8 to 16 days was observed only when TCM199 was used (Fig. 1*J*). Antrum formation (Fig. 1*K*–*M*) was observed at day 8 of IVC. When TCM199 was used as culture medium, 50% of the follicles formed antrum at day 8 and at day 16, 60% of the follicles presented antrum. Differently, at day 8 of IVC 20–30% of the follicles cultured in α-MEM and McCoy media were able to form antrum, and 16 days of IVC was demanded to allow an increase in antrum formation (25–35%) (Fig. 1*N*).

Discussion

Commercial culture media can be successfully used for IVC of different cell types. However, it appears that growth and development of pre-antral follicles require specific substrates in the medium. Initial follicular growth, observed at 8 days of IVC, was positively affected when TCM199 and α-MEM were used. Both media present a similar concentration of glucose (1 g/l), differently from McCoy that presents a concentration three times higher (3 g/l). It has been reported that increased concentration of glucose may promote the down-regulation of proteins responsible to transport sugar in the bovine ovarian tissue, resulting in a deprivation of intracellular glucose and energetic instability (Fladeby *et al.*, 2003). Growth of pre-antral follicles is marked by granulosa cell division, which depends on energy produced by both Krebs and glycolytic cycle, which may lead to a rise in mitochondrial glucose oxidation (Roy & Terada, 1999). Recently, Li *et al.* (2011) have demonstrated that glucose metabolism in cumulus cells maintain the intra-oocyte redox potential. Therefore, we suggest that concentration of glucose in the culture medium

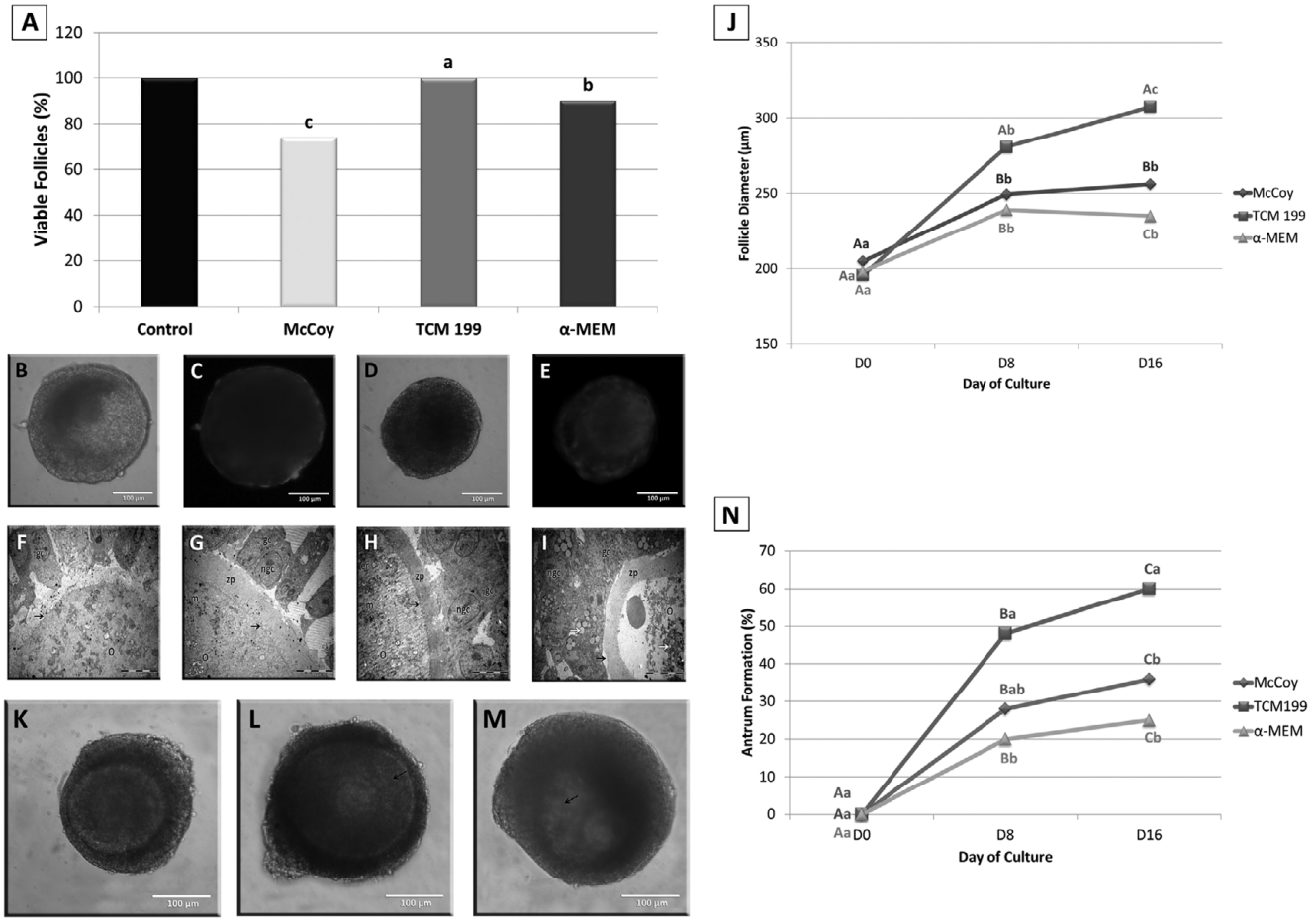

Figure 1 (*A*) Percentages of viable pre-antral follicles before (control) and after 16 days of *in vitro* culture (IVC) in McCoy, TCM199 or α minimum essential medium (α-MEM). (*B–E*) Light microscopic images of normal (*B*) and degenerating (*D*) follicles as confirmed by epifluorescence microscopy of viable calcein-AM stained (*C*) and non-viable ethidium stained (*E*) follicles. (*F–I*) Transmission electron microscopy revealed normal pre-antral follicles after IVC in the presence of α-MEM (*F*) and TCM199 (*G*), whereas McCoy medium lead to granulosa cells detachment from oocyte (*H*) and follicles exhibited signals of vacuolization in the ooplasm (*I*). (*J*) Increase in follicular diameter at days 0, 8 and 16 of IVC. (*K–M*) Follicular antrum formation during IVC in the presence of TCM199. (*N*) Percentage of follicles presenting antrum formation after IVC in the presence of McCoy, TCM199 and α-MEM.

affects the development and oxidative stress in pre-antral follicles. TCM199 is supplemented with adenine sulphate (0.01 g/l) and adenosine (monophosphate and triphosphate, 1 mg/l and 0.24 mg/l respectively), both substrates absent in McCoy medium. Differently from TCM199, α-MEM does not contain adenine sulphate, but adenosine (0.01 g/l). Adenine sulphate, an exogenous precursor of adenosine, has been able to prevent mitochondrial oxidant damage in cardiomyocytes (Xu *et al.*, 2005). Furthermore, Sun *et al.* (2011) reported that adenine sulphate increases superoxide dismutase activity in myocardial cells and reversed ischemia in cardiomyocytes. The advantage of adenine sulphate is its better solubility when compared with adenosine, and its capacity to synthesize adenosine (Sun *et al.*, 2011). Differently from those cultured in presence of McCoy, pre-antral follicles cultured in TCM199 or α-MEM presented integer basement membrane, which may be disrupted under oxidative stress (Rossetto *et al.*, 2009). Based on these previous studies and in our results, we speculated that adenine sulphate may reduce ischemia and oxidative stress in ovarian follicles during *in vitro* culture. Elevated follicular viability was not a parameter to warrant follicular functionality. Whereas follicular viability was higher than 70% after IVC, follicular ability to form antrum ranged of 20 to 60%. Alive cells enclose esterase enzymes able to cleave calcein (de Clerck *et al.*, 1994) and present intact membrane integrity. Not always follicular atresia encompasses membrane damage, and degenerating cells might be able to present active enzymes before death, which may result in an inaccurate method to evaluate follicular function. Therefore, its ability to form antrum

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should be used as a marker of functionality. In summary, the present study showed that during formulation of optimal media for the growth and development of pre-antral follicles not only medium supplementing with growth factors and hormones, but also the composition of the basis medium must be considered. TCM199 appears as an effective medium to promote follicular growth and antrum formation by maintaining follicular morphology, viability and functionality.

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