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Supplementation of culture medium with knockout serum replacement improves the survival of bovine secondary follicles when compared with other protein sources during *in vitro* culture

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Summary

The present study evaluated the effect of knockout serum replacement (KSR), fetal bovine serum (FBS) and bovine serum albumin (BSA) on the viability and growth of bovine secondary follicles cultured *in vitro* for 12 days. To this end, secondary follicles were isolated (185–202 µm) and cultured *in vitro* in TCM-199^{+} medium supplemented with KSR (5% and 10%), FBS (5% and 10%) or BSA (3 mg/ml) at 38.5°C with 5% CO_2 in air. Follicular diameters were evaluated on days 0, 4, 8 and 12. After 12 days of culture, follicular survival analysis was performing by using calcein-AM and ethidium homodimer. Before and after culture, follicles were fixed in paraformaldehyde for histological evaluation. Follicular diameter at different days of culture were compared using the Kruskal–Wallis test, while the percentages of viable follicles were analyzed by chi-squared test ($P < 0.05$). Results showed that follicles cultured in the presence of KSR at both concentrations presented higher follicular survival rates than those cultured in control medium alone or supplemented with FBS or BSA. Conversely, the presence of KSR, BSA or FBS did not increase follicular diameter after 12 days of culture. Histology analysis showed that, among the tested treatments, follicles cultured in the presence of KSR had preserved rounded oocytes, juxtaposed granulosa cells and intact basal membrane. In conclusion, supplementation of culture medium with KSR increases the follicular survival of bovine secondary follicles cultured *in vitro*.

Introduction

The bovine ovary contains thousands of preantral follicles and understanding the mechanisms that control ovarian folliculogenesis *in vitro* can progressively increase the use of oocytes enclosed in preantral follicles in assisted reproductive techniques. The development of an *in vitro* culture protocol that allows better development of secondary follicles up to the antral stage is of utmost importance to provide information about the follicular needs during ovarian development (Silva *et al.*, 2002, 2016). Some *in vitro* studies have shown that preantral follicles are less susceptible to atresia (Meng *et al.*, 2018) and, therefore, these have been commonly used to study follicular development *in vitro* (Almeida *et al.*, 2015; Paulino *et al.*, 2018).

Despite the promising results achieved so far in other species, such as production of embryo after fertilization of *in vitro* grown oocytes (mice: O'Brien *et al.*, 2003; goat: Magalhães *et al.*, 2011), the complete development and production of embryos from oocytes enclosed in bovine preantral follicles has still not been reported (Araújo *et al.*, 2014). According to Beck *et al.* (2018), preantral follicles require for their complete development an efficient culture system that approaches their natural conditions, providing nutrients and energy for cells to ensure their development and growth. Therefore, the formulation of specific medium that meets the metabolic characteristics is of paramount importance (Rossetto *et al.*, 2013).

Fetal bovine serum (FBS) is commonly used as a supplement because of its efficiency in improving cell proliferation, metabolism and differentiation (Cho *et al.*, 2018). FBS also has the ability to maintain cellular activities, providing essential compounds, such as vitamins, binding factors, and being involved in pH buffering or protease inhibitor (Heger *et al.*, 2018). However, in addition to biosafety problems, FBS presents in its composition unknown factors that vary biochemically between different lots, therefore interfering in the standardization of culture medium (van der Valk *et al.*, 2004). Therefore, various studies have emphasized the importance of using serum-free medium during culture of follicles (Park *et al.*, 2013; Motohashi *et al.*, 2017). As an alternative to FBS, knockout serum replacement (KSR) has a

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defined formulation and presents numerous positive effects on cells cultured *in vitro* (Aoshima *et al.*, 2013). KSR supplement consists of essential substances such as lipid-rich albumin, transferrin, insulin, amino acids, vitamins and antioxidants (Garcia-Gonzalo and Belmonte, 2008). Recently, Motohashi *et al.* (2017) demonstrated that oocyte–granulosa cell complexes from mice preantral follicles cultured in medium with either FBS or KSR were able to be fertilized *in vitro* and to deliver live pups. Park *et al.* (2013) also showed that replacement of FBS by KSR did not reduce mice follicle growth or oocyte maturation *in vitro*. Another protein source commonly added to the medium is bovine serum albumin (BSA), which is the most abundant protein in plasma (Majorek *et al.*, 2012). Therefore, it is hypothesized that KSR can be used as a substitute of other protein sources, like FBS, in culture medium of bovine preantral follicles and consequently improves follicular growth and survival *in vitro*.

The aims of the present study were to evaluate the effects of KSR (5% and 10%), FBS (5% and 10%), as well as of BSA (3 mg/ml) on survival and growth of bovine secondary follicles cultured *in vitro* for 12 days.

Materials and methods

Reagents

The culture medium, as well as other products used in this study, were purchased from Sigma Chemicals (St. Louis, MO, USA).

Ovary collection and follicular isolation

For this study, 20 pairs of ovaries from cows were collected from the local slaughterhouse. After collection, the ovaries were washed (approximately 10 s) in 70% alcohol, and were then transported to the laboratory in saline solution containing antibiotics (100 mg/ml penicillin and 100 mg/ml streptomycin) at 4°C for up to 1 h. In the laboratory, fragments of the ovarian cortex (1–2 mm in thickness) were sectioned using a surgical blade under sterile conditions. These fragments were subsequently placed in fragmentation medium (TCM-199) and then isolation of preantral follicles was performed using a stereoscopic microscope (SMZ 645 Nikon, Tokyo, Japan). Secondary follicles were dissected and isolated from the ovarian cortex using 25 gauge (25G) needles.

In vitro *culture of bovine preantral follicles*

Follicles with a visible oocyte surrounded by granulosa cells, an intact basement membrane and no antral cavity were individually cultured in 100 µl medium under mineral oil in Petri dishes (60 × 15 mm, Corning, USA). The culture medium, TCM-199^{+}, was supplemented with ITS (1.0 µg/ml, insulin, 0.55 µg/ml transferrin and 0.5 ng/ml selenium), 50 µg/ml ascorbic acid, 3 mM glutamine, 2 mM hypoxanthine, 10 mg/ml penicillin and 10 mg/ml of streptomycin. Secondary follicles were randomly cultured in TCM-199^{+} alone (culture control) or supplemented with KSR (5% or 10%, Invitrogen, São Paulo, Brazil), FBS (5% or 10%) or BSA (3 mg/ml). The follicles were cultured at 38.5°C with 5% CO_2 for 12 days. Medium filtration was performed after adding the supplements. Every 2 days of culture, 60 µl of medium was replaced with fresh medium.

Morphological and viability evaluation of cultured follicles

At days 0, 4, 8 and 12 of culture, morphologically normal follicles with well organized granulosa cells, and intact oocyte and basement membrane were observed. Finally, two perpendicular measurements were performed on normal follicles using an inverted microscope with the software NIS Elements 2.4 (Nikon Instruments Inc., Japan). The presence of an antral cavity in cultured follicles was also evaluated.

To confirm the results of morphological analyzes, follicles that were considered normal were further evaluated for their survival using a more accurate method based on fluorescent probes. After culturing, the follicles ($n = 8$ per treatment) were stained with 4 mM calcein-AM and 2 mM ethidium homodimer (Molecular Probes, Invitrogen, Karlsruhe, Germany) at 37°C for 15 min. Subsequently, the follicles underwent three washes in TCM-199 and were examined under a fluorescence microscope (Nikon, Eclipse, TS 100, Japan). The oocytes and granulosa cells were considered viable if the cytoplasm was positively stained a green colour (calcein-AM) and were not stained red by the ethidium homodimer (van den Hurk *et al.*, 1998; Vasconcelos *et al.*, 2013).

Evaluation of follicle morphology by classical histology

Both fresh and 12-day cultured follicles were fixed for 24 h in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) and then, dehydrated in a graded series of alcohol (70, 80, 90 and 100%), diaphanized with xylene and then embedded in paraffin wax. For each group of follicles, sections (5 µm thick) were performed and stained with haematoxylin–eosin. All sections were examined and the follicles were evaluated under a light microscope (Nikon, Japan). To evaluate the follicular morphology, follicles presenting well organized granulosa cells, spherical oocytes with a nucleus, and intact basement membrane were considered to be normal. For each treatment, five follicles were histologically evaluated.

Statistical analysis

For analysis of follicular growth, the data were expressed in mean (± standard error of the mean, SEM) and submitted to analysis of variance (ANOVA); means were compared using the Kruskal–Wallis test using Stat View 5.0 (SAS Institute Inc., Cary, NY, USA). After 12 days of culture, percentages of follicular survival were analyzed using the chi-squared test. The differences were considered significant when P-values were < 0.05.

Results

Effects of different protein sources on follicular growth and viability

In general, a progressive increase in follicular diameter was observed in follicles cultured *in vitro*. After 8 days, follicles cultured in TCM-199^{+} alone or supplemented with 5% and 10% KSR, as well as 10% FBS had higher diameters than those follicles at day 0. After 12 days, follicles cultured in all media had higher diameters when compared with follicles at day 0 (Table 1). However, when comparisons among media were performed after 4, 8 or 12 days of culture, the presence of KSF (5 or 10%), FBS (5 or 10%) or BSA did not influence follicular growth *in vitro*. Antrum formation was rarely observed during culture.

After 12 days, follicles cultured with 5% or 10% KSR had higher rates of viability ($P < 0.05$) when compared with those cultured in control medium alone or supplemented with FBS or BSA (Fig. 1). Conversely, follicles cultured in the presence of BSA and 10% FBS showed a similar rate of follicular survival when compared with those cultured in control medium. In addition,

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Table 1. Effects of different protein sources on the diameters (μm) of bovine secondary follicles during 12 days of culture

	Day 0	Day 4	Day 8	Day 12
TCM-199	191.8 ± 5.8^{a}	209.6 ± 6.2^{a,b}	223.6 ± 7.6^{b}	227.2 ± 7.6^{b}
BSA	193.8 ± 5.1^{a}	205.3 ± 5.9^{a,b}	214.9 ± 6.4^{a,b}	229.1 ± 6.5^{b}
FBS 5%	185.8 ± 6.3^{a}	192.2 ± 8.3^{a,b}	205.7 ± 7.7^{a,b}	220.5 ± 8.6^{b}
FBS 10%	202.3 ± 5.2^{a}	208.7 ± 5.3^{a,b}	227.9 ± 6.8^{b}	236.0 ± 8.5^{b}
KSR 5%	195.9 ± 4.8^{a}	212.6 ± 5.2^{a,b}	219.4 ± 5.7^{b}	226.0 ± 5.4^{b}
KSR 10%	190.4 ± 5.2^{a}	206.4 ± 5.5^{a,b}	215.0 ± 5.5^{b}	220.8 ± 6.0^{b}

^{a,b}Means difference between days of culture within each treatment.

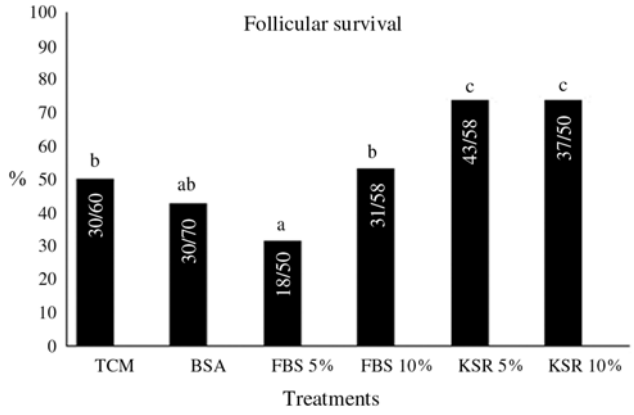

Figure 1. Survival rate of bovine secondary follicles cultured in TCM-199^{+} alone or supplemented with BSA, FBS (5% or 10%) KSR (5% or 10%) after 12 days of culture *in vitro*. $^{a-c}$Lowercase letters show significant differences between treatments.

follicles cultured in medium with 5% FBS had the lowest viability rate among treatments.

To confirm follicle viability, the fluorescence analysis showed that normal follicles after 12 days of culture had homogeneous granulosa cells and oocytes that were positively stained for calcein (green). In some follicles, especially those cultured in TCM-199 alone or supplemented with BSA or 10% KSR had stromal/thecal cells around the follicles stained positively with ethidium homodimer (red) (Fig. 2).

Histological evaluation of secondary follicles before and after 12 days of culture

Figure 3 shows that secondary follicles within uncultured ovarian tissue had centralized oocyte various layers of well organized granulosa cells (Fig. 3A). Figure 3B shows that, after isolation, secondary follicles keep their follicular structure with intact oocyte, granulosa cells and basement membrane. After 12 days of culture in the presence of 5% KSR, secondary follicles increased their diameter and maintained the morphology of their oocyte, granulosa and theca cells (Fig. 3C). Figure 3D shows the morphology of degenerated follicles with rupture of basal membrane, follicle retraction and disorganized granulosa cells, in addition to absence or oocyte degeneration.

Discussion

This study shows that supplementation of culture medium with 5% or 10% KSR increases the survival of bovine secondary follicles when compared with follicles cultured in the presence of FBS or BSA after 12 days of culture *in vitro*. Previously, Fujihara *et al.* (2012) reported that feline preantral follicles cultured in medium supplemented with 10% KSR had higher viability than those cultured in medium with added FBS. In mice, oocyte–granulosa cell complexes from preantral follicles cultured in medium with either FBS or KSR achieved fertilization *in vitro* and post-implantation development (Motohashi *et al.*, 2017). Park *et al.* (2013) also showed that replacement of FBS by KSR did not reduce mice preantral follicle growth or oocyte maturation *in vitro*. Comparing the effects of BSA and FBS during culture of bovine preantral follicles, Bernuci *et al.* (2013) reported a greater percentage of viable follicles, antrum formation, and of retrieved healthy cumulus–oocyte complexes in follicles cultured with BSA. Supplementation of culture medium with 5% KSR also improved the rates of porcine oocyte maturation and blastocyst formation after *in vitro* fertilization (Jin *et al.*, 2018). In other studies, the supplementation of culture medium with 5% KSR enhanced the *in vitro* viability of porcine blastocysts (Sakurai *et al.*, 2015). KSR has a well defined and standardized composition and can be widely used as a direct substitute of FBS or BSA in some existing protocols, as KSR presents a mixture of small organic molecules (amino acids, vitamins and antioxidants), trace elements and three proteins, namely insulin, transferrin and lipid-rich albumin in its composition, which can help to maintain follicular viability (Garcia-Gonzalo and Belmonte, 2008; Price *et al.*, 1998).

Differently from the results obtained with the presence of 5% KSR, the supplementation of culture medium with BSA or FBS did not improve secondary follicles viability *in vitro*. Consistent with the present results, Hulshof *et al.* (1995) found that the FBS did not influence follicular viability, but decreased oocyte development and caused loss of basement membrane integrity in cultured follicles (Thomas *et al.*, 2001). The addition of 10% FBS to medium used for cryopreservation of zebrafish ovarian follicles did not allow the achievement of successful cryopreservation indicating a compromised metabolic status (Zampolla *et al.*, 2012). Although FBS is commonly used in the *in vitro* production of bovine embryos (Del Collado *et al.*, 2014, 2015), the presence of FBS in culture medium may cause a divergence in results during *in vitro* production of embryos (Brunner *et al.*, 2010). During *in vitro* oocyte maturation, the addition of BSA failed to support cumulus expansion for bovine or hamster cumulus–oocyte complexes and did not support cumulus expansion or completion of meiosis I in bovine COCs (Leibfried-Rutledge *et al.*, 1986).

This study shows that follicles cultured with 5% KSR have oocyte, granulosa cells and basement membrane histologically well preserved. Cadoret *et al.* (2017) reported the importance of preservation of follicular structure after culture *in vitro* and that the maintenance of integrity of basement membrane is essential to increase the follicle diameter, as well as the number of follicular cells. Moreover, they also noticed that some of the cultured follicles did not survive until the end of the culture period, mainly due to rupture of the basement membrane and opening of the follicular structure.

In this study, the protein sources tested (KSR, FBS or BSA) did not improve preantral follicle growth and antrum formation was rarely observed after 12 days of culture. Bernuci *et al.* (2013) also did not detect differences in diameters of preantral follicles cultured in the presence of either BSA or FBS, but they showed that follicles cultured for 21 days with BSA had a higher rate of antrum

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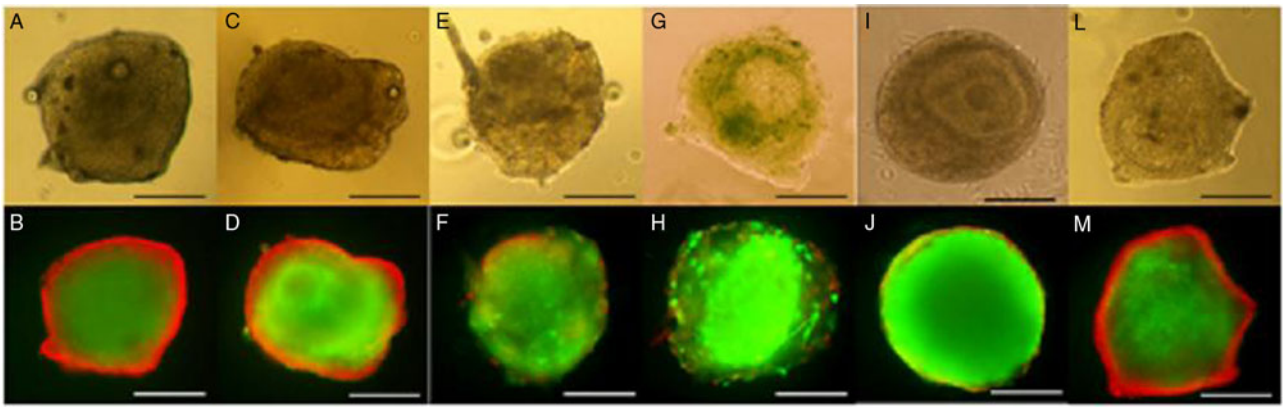

Figure 2. Secondary follicles cultured in TCM-199^{+} alone (*A*, *B*) or supplemented with BSA (*C*, *D*), 5% FBS (*E*, *F*), 10% FBS (*G*, *H*), 5% KSR (*I*, *J*) or 10% KSR (*L*, *M*) for 12 days and evaluated by light microscopy and after staining with calcein-AM (green) and ethidium homodimer-1 (red). Considering that only surrounding stromal cells were stained with ethidium homodimer-1 in some follicles, all of these were considered viable. Scale bars represent 100 µm.

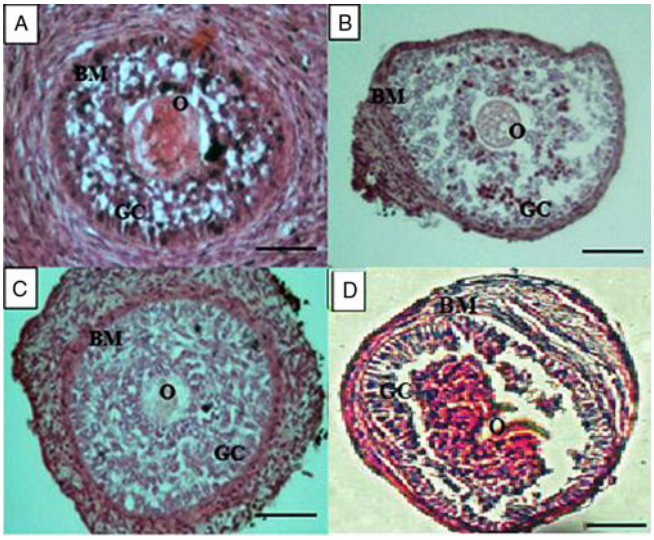

Figure 3. Characteristics of secondary follicles within ovarian tissue (*A*), after being isolated (*B*), and cultured *in vitro* for 12 days in the presence of 5% KSR (*C*). A degenerated secondary follicle after 12 days of culture *in vitro* is shown in (*D*). Basal membrane (BM), oocyte (O) and granulosa cells (GC). Scale bars represent 50 µm.

formation than follicles cultured in the presence of FBS. These data emphasize that antrum formation *in vitro* depends on longer culture periods, as preantral follicles need around 70 days to grow up to antral stages (Gougeon, 2010). In addition, the replacement of FBS by KSR did not influence the number of follicles that formed a pseudo-antrum cavity after *in vitro* culture (Park *et al.*, 2013).

In conclusion, after 12 days of *in vitro* culture, the presence of 5% or 10% KSR increases the survival and maintained the morphology of oocyte, granulosa and theca cells compared with follicles cultured in the presence of FBS or BSA. However, the presence of these supplements in culture medium was not able to increase follicular diameter.

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Conflicts of interest. The authors declare that there are no conflicts of interest.

Ethical standards. The authors declare that all procedures were performed according to national and institutional guides on the care and use of animals.

References

Almeida AP, Magalhães-Padilha DDM, Araújo VR, Costa SLD, Chaves RN, Lopes C AP, Donato MA, Peixoto CA, Campello CC, Junior JB and Figueiredo JR (2015) Effect of sequential medium with fibroblast growth factor-10 and follicle stimulating hormone on *in vitro* development of goat preantral follicles. *Anim Reprod Sci* **152**, 32–8.

Aoshima K, Baba A, Makino Y and Okada Y (2013) Establishment of alternative culture method for spermatogonial stem cells using knockout serum replacement. *PLoS One* **8**, 77715.

Araújo VR, Gastal MO, Figueiredo JR and Gastal EL (2014) *In vitro* culture of bovine preantral follicles: a review. *Reprod Biol Endocrinol* **12**, 78.

Beck K, Singh J, Dar MA and Anzar M (2018) Short-term culture of adult bovine ovarian tissues: chorioallantoic membrane (CAM) vs. traditional *in vitro* culture systems. *Reprod Biol Endocrinol* **16**, 1–10.

Bernuci MP, Rosa-e-Silva ACJS, Batista L, Garcia C, Campos JO and Sá MFS (2013) Effect of fetal calf serum and bovine serum albumin on *in vitro* development of bovine preantral follicles during three-dimensional culture. *Fertil Steril* **100**, 168–9.

Brunner D, Frank J, Appl H, Schoffl H, Pfaller W and Gstraunthaler G (2010) Serum-free cell culture: the serum-free media interactive online database. *Altex* **27**, 53–62.

Cadoret V, Frapsauce C, Jarrier O, Maillard V, Bonnet A, Locatelli Y, Royère D, Monniaux D, Guérif F and Monget P (2017) Molecular evidence that follicle development is accelerated *in vitro* compared with *in vivo*. *Reproduction* **153**, 493–508.

Cho H, Lee A and Kim K (2018) The effect of serum types on chondrogenic differentiation of adipose-derived stem cells. *Biomater Res* **22**, 6.

Del Collado M, Saraiva NZ, Lopes FL, Cruz MH, Gaspar RC, Oliveira CS, Perecin F and Garcia JM (2014) Effects of reduction or replacement of fetal calf serum by other compounds during *in vitro* maturation of bovine oocytes. *Pesq Vet Bras* **34**, 689–94.

Del Collado M, Saraiva NZ, Lopes FL, Gaspar RC, Padilha LC, Costa RR, Rossi GF, Vantini R and Garcia JM (2015) Influence of bovine serum albumin and fetal bovine serum supplementation during *in vitro* maturation on lipid and mitochondrial behaviour in oocytes and lipid accumulation in bovine embryos. *Reprod Fert Dev* **28**, 689–94.

Fujihara M, Comizzoli P, Wildt DE and Songsasen N (2012) Cat and dog primordial follicles enclosed in ovarian cortex sustain viability after *in vitro* culture on agarose gel in a protein-free medium. *Reprod Domest Anim* **47**, 102–8.

Garcia-Gonzalo FR and Belmonte JCL (2008) Albumin-associated lipids regulate human embryonic stem cell self-renewal. *PLoS One* **3**, 1384.

https://doi.org/10.1017/S0967199419000583 Published online by Cambridge University Press

Gougeon A (2010) Human ovarian follicular development: from activation of resting follicles to preovulatory maturation. *Ann Endocrinol (Paris)* **71**, 132–43.

Heger IJ, Froehlich K, Pastuscheck J, Schmidt A, Baer C, Mmrowka R, Backsch C, Schleuβner E, Markert UR and Schmidt A (2018) Human serum alters cell culture behavior and improves spheroid formation in comparison to fetal bovine serum. *Exp Cell Res* **365**, 57–65.

Hulshof SCJ, Figueiredo JR, Beckers JF, Bevers MM, van der Donk JA and van den Hurk R (1995) Effects of fetal bovine serum, FSH and 17β–estradiol on the culture of bovine preantral follicles. *Theriogenology* **44**, 217–26.

Jin JX, Lee S, Setyawan EMN, Taweechaipaisankul A, Kim GA, Han HJ, Ahn C and Lee BC (2018) A potential role of knockout serum replacement as a porcine follicular fluid substitute for *in vitro* maturation: lipid metabolism approach. *J Cell Physiol* **233**, 6984–95.

Leibfried-Rutledge ML, Critser ES and First NL (1986) Effects of fetal calf serum and bovine serum albumin on *in vitro* maturation and fertilization of bovine and hamster cumulus–oocyte complexes. *Biol Reprod* **35**, 850–7.

Magalhães DM, Duarte AB, Araújo VR, Brito IR, Soares TG, Lima IM, Lopes CA, Campello CC, Rodrigues AP and Figueiredo JR (2011) *In vitro* production of a caprine embryo from a preantral follicle cultured in media supplemented with growth hormone. *Theriogenology* **75**, 182–8.

Majorek K, Porebski PJ, Dayal A, Zimmermman MT, Jablonska K, Stewart AJ, Chruszcz M and Minor W (2012) Structural and immunologic characterization of bovine, horse, and rabbit serum albumins. *Mol Immunol* **52**, 174–82.

Meng L, Jan SZ, Hamer G, van Pelt AM, van der Stelt I, Keijer J and Teerds KJ (2018) Preantral follicular atresia occurs mainly through autophagy, while antral follicles degenerate mostly through apoptosis. *Biol Reprod* **99**, 853–63.

Motohashi HH, Taniguchi R, Sakamoto J, Sankai T and Kada H (2017) Live, full-term mouse pups from oocytes grown and matured *in vitro* with serum substitutes. *Reprod Biol* **17**, 180–4.

O'Brien MJ, Pendola JK and Eppig JJ (2003) A revised protocol for *in vitro* development of mouse oocytes from primordial follicles dramatically improves their developmental competence. *Biol Reprod* **68**, 1682–6.

Park YH, Gong SP, Kim HY, Kim GA, Choi JH, Ahn JY and Lim JM (2013) Development of a serum-free defined system employing growth factors for preantral follicle culture. *Mol Reprod Dev* **80**, 725–33.

Paulino LRFM, Cunha EV, Silva AWB, Souza GB, Lopes EPF, Donato MAM, Peixoto CA, Matos-Brito BG, van den Hurk, R and Silva JRV (2018) Effects of tumour necrosis factor-alpha and interleukin-1 beta on *in vitro* development of bovine secondary follicles. *Reprod Dom Anim* **53**, 997–1005.

Price PJ, Goldsborough MD and Tilkins ML. Embryonic stem cell serum replacement: U.S. Patent, 1998, International Publication Number: WO/1998/30679 (International Application Number: PCT/US1998/000467).

Rossetto R, Saraiva MV, Dos santos RR, Da Silva CM, Faustino LR, Chaves RN, Brito IR, Rodrigues GQ, Lima IM, Donato MA, Peixoto CA and De Figuereido JR (2013) Effect of medium composition on the *in vitro* culture of bovine pre-antral follicles: morphology and viability do not guarantee functionality. *Zygote* **21**, 125–8.

Sakurai M, Suzuki C and Yoshioka K (2015) Effect of knockout serum replacement supplementation to culture medium on porcine blastocyst development and piglet production. *Theriogenology* **83**, 679–86.

Silva JRV, Ferreira LAM, Costa SHF and Figueiredo JR (2002) Morphological features and control of follicular growth during folliculogenesis in domestic ruminants. *Cienc Anim* **12**, 105–17.

Silva JRV, van den Hurk R and Figueiredo JR (2016) Ovarian follicle development *in vitro* and oocyte competence: advances and challenges for farm animals. *Domest Anim Endocrinol* **55**, 123–35.

Thomas FH, Leask R, Srsen V, Riley SC, Spears N and Telfer EE (2001) Effect of ascorbic acid on health and morphology of bovine preantral follicles during long-term culture. *Reproduction* **122**, 487–95.

van den Hurk R., Spek ER, Hage WJ, Fair T, Ralph JH and Schotanus K (1998) Ultrastructure and viability of isolated bovine preantral follicles. *Hum Reprod* **4**, 833–41.

van der Valk J, Mellor D, Brands R, Fischer R, Gruber F, Gstraunthaler G, Hellebrekers L, Hyllner J, Jonker FH, Prieto P, Thalen M and Baumans V (2004) The humane collection of fetal bovine serum and possibilities for serum-free cell and tissue culture. *Toxicol In Vitro* **18**, 1–12.

Vasconcelos GL, Saraiva MVA, Costa JJN, Passos MJ, Silva AWB, Rossi, RODS, Portela AMLR, Duarte ABG, Magalhães-Padilha DM, Campelo CC, Figueiredo JR, van den Hurk R and Silva JRV (2013) Effects of growth differentiation factor-9 and FSH on *in vitro* development, viability and mRNA expression in bovine preantral follicles. *Reprod Fertil Dev* **25**, 1194–203.

Zampolla T, Rawson DM and Zhang T (2012) The use of fetal bovine serum for cryopreservation of stage III zebrafish (*Danio rerio*) ovarian follicles. *Cryo Lett* **33**, 435–42.