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Influence of follicle-stimulating hormone concentrations on the integrity and development of bovine follicles cultured *in vitro*

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Summary

This study investigated the *in vitro* culture of bovine follicles included in ovarian tissue for 2 or 6 days (D2 or D6), with the addition of different concentrations of follicle-stimulating hormone (FSH) (0, 10, 50, 100 or 200 ng/ml). Data were compared for follicular development, morphological integrity and diameter of follicles and oocytes. Ovaries ($n = 10$) from Nelore cows ($n = 5$) were divided into fragments ($n = 11$ per ovary) and were immediately fixed in Bouin's solution (D0) or were individually cultured for 2 or 6 days in one of the described concentrations of FSH and then processed for histology. Compared with the rates of follicular development at D2 for minimal essential medium (MEM) (75.0%) and 50 ng/ml of FSH (71.1%), the best rates of follicular development at D2 were obtained with 10 (84.7%), 100 (87.5%) and 200 ng/ml of FSH (85.0%; $P < 0.05$). After 6 days of cultivation, there were no differences among treatments regarding follicular growth. The morphological integrity of preantral follicles was better maintained by 100 ng/ml FSH for 2 and 6 days of cultivation (51.2 and 40.4%, respectively; $P < 0.05$) than that for MEM (D2: 30.9%, D6: 20.8%), 10 (D2: 39.2%, D6: 22.8%), 50 (D2: 30.4%, D6: 28.8%) and 200 ng/ml FSH (D2: 45.2%, D6: 36.8%). FSH at 100 ng/ml provided the highest mean diameter averages: 34.5 ± 10.8 μm at D2 and 33.2 ± 12.5 μm at D6 ($P < 0.05$). We concluded that the medium supplemented with 100 ng/ml FSH during *in vitro* culture provided appropriate conditions for the development and morphological integrity of preantral follicles in cattle.

Introduction

Over recent decades, it has become common to investigate the mechanisms involved in follicular development among mammalian species. The reserve preantral follicles in the ovary are abundant (≥90%), but a small portion reaches the ovulation route to be fertilized by a sperm (Gomes *et al.*, 2015). Understanding these mechanisms *in vivo* and *in vitro* makes it possible to improve the *in vitro* culture system for ovarian follicles, as the regulation of early follicular development is unknown (Gougeon, 1996; Farin *et al.*, 2007; Araújo *et al.*, 2014). The *in situ* culture mimics the *in vivo* physiological events by featuring specific medium supplemented with substances that enable the activation, development, and maintenance of follicular integrity and adjacent ovarian stromal cells. Therefore, the *in situ in vitro* culture of follicles is one of the most effective tools available to unlock folliculogenesis and enable assisted animal reproduction (Gomes *et al.*, 2015; Silva *et al.*, 2016).

The follicular growth in the ovaries is established by the presence of gonadotropins and growth factors such as intraovarian regulators (Fortune, 2003). The molecular mechanisms and factors produced by the ovaries have been studied regarding performance in preantral follicles. Recently, findings have identified substances that allow survivability, maturation and follicular growth, and reduced follicular death. Although this technique has satisfactory results, many studies have addressed attempts to use combinations of different substances, such as growth factors, hormones, and antioxidants (Araújo *et al.*, 2014; Silva *et al.*, 2016). These associated components act to stimulate follicular growth and survival, and inhibit atresia processes.

Gonadotropic hormones are responsible for regulating the development of follicles in the ovaries. So far, it is believed that the early stages of ovarian folliculogenesis usually progress independent of gonadotrophins, while the late stages regulate its development by the presence of gonadotrophins (Salomon *et al.*, 2018). Therefore, cases are depicting the stimulating effect of follicle-stimulating hormone (FSH) on follicular activation. The respective receptors are

arranged in a specific manner, acting in synchrony and promoting stimulation and/or inhibitory effects (Almeida *et al.*, 2011). FSH is involved in stimulating steroidogenesis, acting in the *in vitro* proliferation and differentiation of granulosa cells, and FSH is apparently involved in early folliculogenesis (Wandji *et al.*, 1996; Kreeger *et al.*, 2005). FSH is also related to the development and viability of follicles isolated in different concentrations and species (goats: Magalhães *et al.*, 2011; sheep: Rodrigues *et al.*, 2010; bovine: McLaughlin *et al.*, 2010).

FSH is one of the important substances in the process of folliculogenesis. The hypothesis is that the specific concentration of FSH may affect the activation, growth, and integrity of preantral cattle follicles. Therefore, the objective of this study was to assess the concentration curve of FSH; 10, 50, 100 and 200 ng/ml) in the *in vitro* cultivation of the development, survival and integrity of preantral follicles bovine.

Materials and methods

All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted during the study.

Collection and transport of ovaries

Ovaries ($n = 10$) were collected from a local slaughterhouse (latitude 23°17′34″ and longitude 51°10′24″) from five adult *Bos indicus* females that were cyclical, judging by the corpus luteum presence, and in body condition (body score of 2.5 to 3.5 on a scale of 0 to 5). After recovery, the ovaries were washed in 70% ethanol and buffer solution (phosphate-buffered saline (PBS); Embriolife®, Vitrocell, Campinas, São Paulo, Brasil), processed, and transported to a temperature-controlled laboratory (20–24°C; approximately 30 km). The transport of bovine ovaries used minimal essential medium (MEM; Gibco BRL, Rockville, MD, USA; osmolarity 300 mOsm/l, pH 7.2) supplemented with 200 mg/ml penicillin and 200 mg/ml streptomycin.

Cultivation of preantral follicles and experimental protocol

The pair of ovaries from each animal was carefully processed; surrounding tissue and ligaments were removed, and then the ovary was sectioned longitudinally. The medulla, large antral follicles and corpora lutea were removed. Subsequently, the cortex of the ovary was divided into fragments of approximately $3 \times 3 \times 1$ mm. (Fig. 1) For each animal, a fragment was randomly selected and immediately fixed in Bouin's solution (treatment control non-cultivated, D0). The remaining fragments of the ovarian cortex were cultured individually in 1 ml aliquots of culture medium in 24-well culture plates in an incubator at 38.5° C in an atmosphere of 5% CO_2 in air and saturated humidity. The control culture comprised MEM, Gibco BRL, Rockville, MD, USA; osmolarity 300 mOsm/l, pH 7.2) supplemented (MEM+) with insulin–transferrin–selenium (ITS) (insulin 6.25 mg/ml, transferrin 6.25 mg/ml and selenium 6.25 ng/ml), 0.23 mM pyruvate, 2 mM glutamine, 2 mM hypoxanthine, 1.25 mg/ml bovine serum albumin (BSA Gibco BRL, Rockville, MD, USA), 20 UI/ml penicillin and 200 mg/ml streptomycin. For treatments, the MEM^+ was supplemented with porcine pituitary FSH (Folltropin®, Bioniche Canada Inc., Ontario, Canada) at concentrations of 10, 50, 100 or 200 ng/ml (FSH 10, FSH 50, FSH 100, FSH 200). Ten fragments of the ovarian cortex of each animal were cultured in medium and tested at 2 (D2) or 6 (D6) days. At each interval of 2 days, the culture medium was replaced by fresh medium. The cultivation period used was based on published studies on the activation time course of primordial follicles in other species (Braw-Tal & Yossefi, 1997; Fortune *et al.*, 1998).

Histological processing

For the analysis of ovarian morphology and ovarian follicles, control ovarian cortex fragments (non-cultivated) were cultured for 2 or 6 days and fixed by immersion in Bouin's solution for 24 h. After fixation the fragments, the tissues were kept in 70% ethanol for 24 h and dehydrated in a graded series of solutions of increasing ethanol, subjected to clarified and cleared in xylene, and embedded in paraffin for preparation of blocks for histological analysis. Subsequently, each block was sectioned to a thickness of 5 µm with an interval of five sections of tissue with a rotary microtome (Leica®, Wetzlar, Germany) for mounting microscope slides (Andrade *et al.*, 2012; Max *et al.*, 2017). The slides were stained with periodic acid–Schiff (PAS) and haematoxylin (Gomes *et al.*, 2018) stains. For PAS staining, the tissue slides were first deparaffinized with xylene and then rehydrated using 70% ethanol prior to staining with periodic acid for 5 min and the Schiff reaction for 10 min.

Morphologic analysis and assessment of in vitro follicular growth

All sections were examined using optical microscopy (×10 and ×40; Nikon, Tokyo, Japan). Follicles were ranked according to the following detailed specifications (Hulshof *et al.*, 1994). Preantral follicles were classified as: (1) primordial (one layer containing somatic cells, known as granulosa cells, flat or flattened around the oocyte) or in development; (2) primary (a single layer of cuboidal granulosa cells around the oocyte); or (3) secondary (two or more layers of granulosa cells cubic). To evaluate the activation and follicle growth, the follicles were quantified at different stages of development (primordial, primary and secondary) in control and after *in vitro* cultivation in different treatments.

Follicles also were classified according to morphology integrity according to the structure, in intact or degenerated. The follicle present was called morphologically intact when it had a regular nucleus and was surrounded by granulosa cells arranged in discrete layers; the follicle was called degenerated when it was huddled with a pyknotic nucleus and surrounded by granulosa cells isolated from disorganized basement membrane (Búfalo *et al.*, 2016). Then, approximately 250 follicles per treatment were studied for the duration of cultivation. To prevent recounts, preantral follicles were counted only in the section in which the nucleus of the oocyte was observed. To evaluate the growth rates and follicular activation, only intact follicles were considered, and the percentages of primordial, primary and secondary follicles were calculated after 0 (control), 2 or 6 days of culture at varying concentrations of the culture medium.

Morphometry analysis of preantral follicles cultured in vitro

For the analysis of follicles and cultured oocyte diameters, five morphologically intact preantral follicles were observed per treatment (MEM+, FSH 10, FSH 50, FSH 100, FSH 200) among the five replicates, and images were captured. In total, 250

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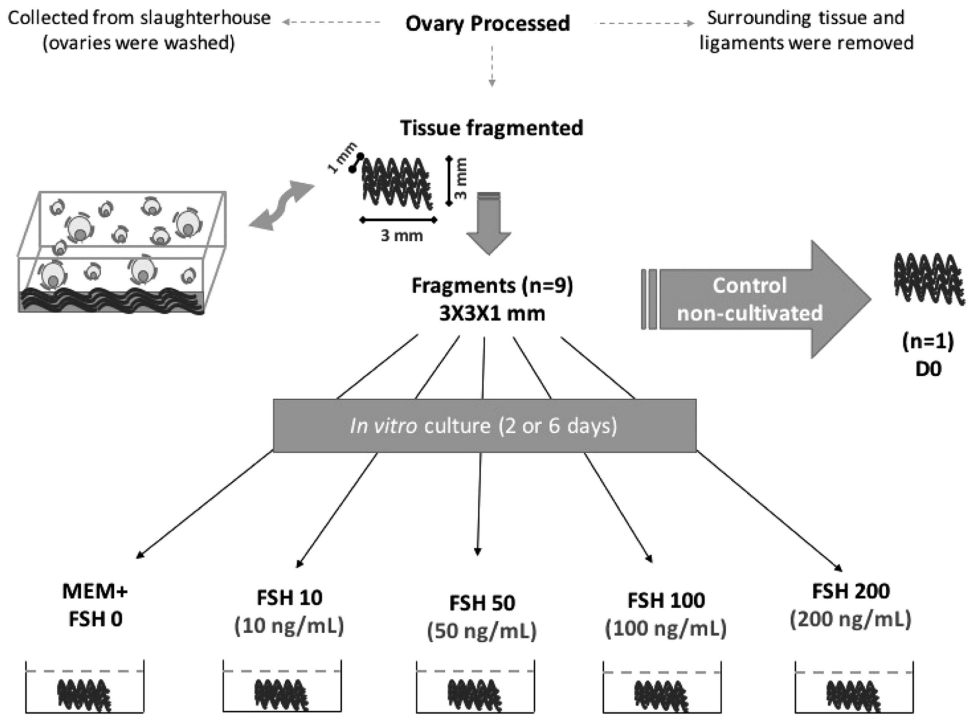

Figure 1. Experimental protocol used for *in vitro* culture of bovine preantral follicles in control medium (MEM+) or medium containing follicle-stimulating hormone (FSH).

preantral follicles were evaluated in the cultured treatments at 2 days ($n = 125$) and 6 days ($n = 125$). The measurements of follicles and oocytes were performed according to Silva-Buttkus *et al.* (2008), and the oocyte and follicular diameters were calculated from the arithmetic mean of two perpendicular measurements using the Motic Plus 2.0 software program.

Statistical analysis

The data were initially submitted to normality tests (Shapiro–Wilk) and homogeneity of variance (Bartlett). The mean number of morphologically intact preantral follicles, primordial follicles and developing follicles obtained in the control treatments and different concentrations of FSH cultured for 2 or 6 days were subjected to analysis of variance (ANOVA) and Tukey's test ($P \leq 0.05$). All analyses were performed with the Action 3.1 software, version of R 3.0.2 (Campinas, SP, Brazil), and the values were considered statistically significant when $P \leq 0.05$.

Results

Morphologic analysis and assessment of in vitro *follicular growth*

In total, 2250 preantral follicles were evaluated in the experiment, with 250 follicles assessed per treatment (Fig. 2*A*). Considering the stage of development, 772 were classified as primordial follicles and 1478 as developing follicles (Fig. 2*B*, *D*). For the histological analysis, the integrity and degeneration of follicles were observed during the *in vitro* culture of ovarian cortex (Fig. 2*B*, *C*). Normal stromal tissue was observed between follicles (Fig. 2*E*).

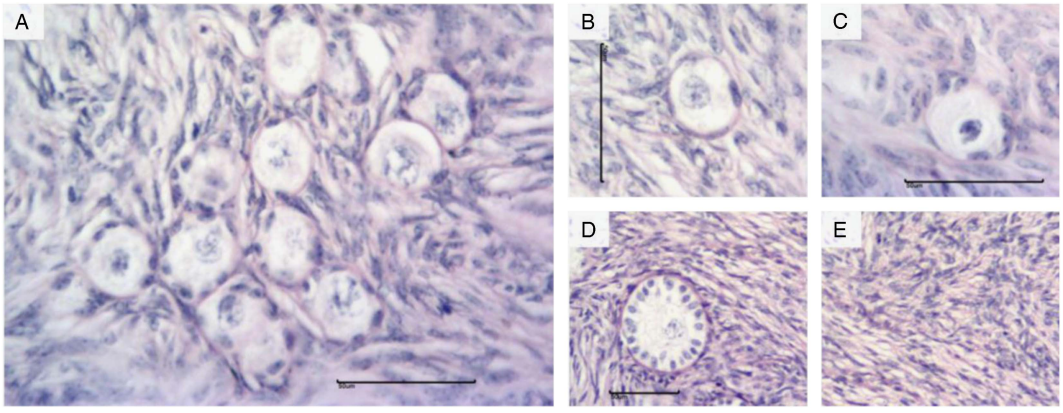

Figure 2. Histological illustration of bovine preantral follicle morphology. (*A*) Preantral follicles. (*B*) Intact primordial follicle. (*C*) Degenerated primordial follicle. (*D*) Intact development follicle. (*E*) Normal stroma. The fragments were stained using periodic acid–Schiff (PAS) and haematoxylin stains. Scale bar represents 50 μm.

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The non-cultivated control (D0) contained largely intact primordial follicles (90.4%; Fig. 3) and a small portion of developing follicles (9.6%; Fig. 4). The distribution of intact primordial follicles and intact developing follicles present in the *in vitro* cortical tissue at 2 or 6 days cultivation in the presence or absence of FSH is shown in Fig. 3 and Fig. 4, respectively.

In general, after the start of cultivation, the proportion of primordial follicles was reduced between the different treatments. Therefore, there was an increased percentage of developing follicles. In all treatments for 2 or 6 days, the mean percentage of primordial follicles decreased compared with the non-cultured control, however there was no significant difference between the groups ($P > 0.05$; Fig. 3).

Regarding the development of primordial follicles, the ovarian tissue supplemented with 10, 100 or 200 ng/ml FSH for 2 days efficiently led to the development of preantral follicles. However, regarding initiation, the concentrations of 100 and 200 ng/ml FSH were better than 10 ng/ml and MEM + ($P < 0.05$; Fig. 4). The percentage of follicles in developing D6 did not obtain significant differences between treatments (MEM + , 10, 50, 100 or 200 ng/ml FSH; $P < 0.05$; Fig. 4).

Survival rates and follicular integrity in the two-dimensional culture are shown in Fig. 5. The percentages of intact follicles were significantly higher in the 100 ng/ml FSH group cultured for 2 or 6 days than those in the other treatments ($P \leq 0.05$).

Morphological changes identified in the atretic preantral follicles were mainly characterized as retracted oocyte, pyknotic nucleus disorganized granulosa cells and low cell density.

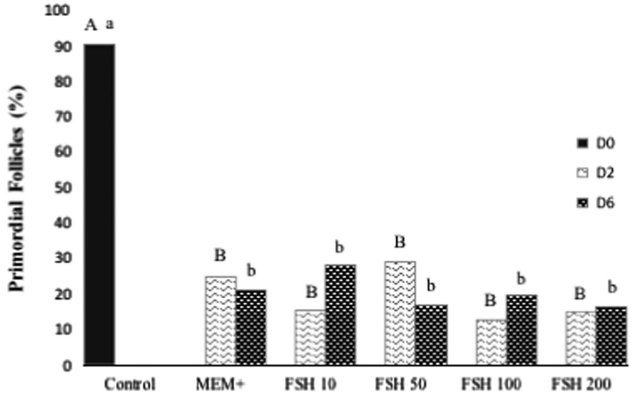

Figure 3. Percentage of intact follicles from female *Bos indicus* at early stage (primordial) and non-cultured ovarian tissue (control, day 0) and after *in vitro* culture for 2 or 6 days in MEM + or MEM + supplemented with different concentrations of FSH (10, 50, 100 or 200 ng/ml). [A,B]Values within a treatment (culture day – D2) with different superscripts differ significantly at $P < 0.05$ (between treatments – Control, MEM + , FSH 10, FSH 50, FSH 100 and FSH 200). [a,b]Values within a treatment (culture day – D6) with different superscripts differ significantly at $P < 0.05$ (between treatments – Control, MEM + , FSH 10, FSH 50, FSH 100 and FSH 200).

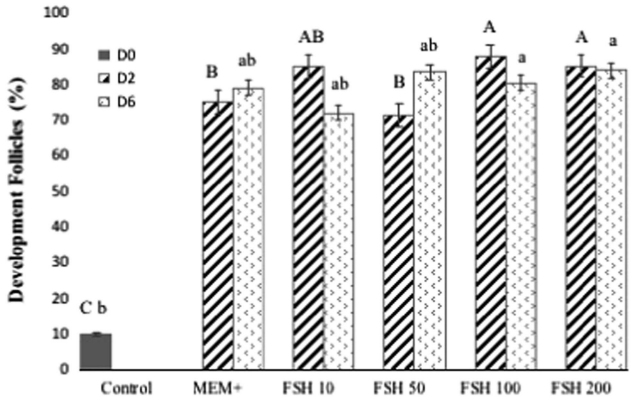

Figure 4. Percentage of intact follicles from female *Bos indicus* at development (primary and secondary) in non-cultured ovarian tissue (control, day 0) and after *in vitro* culture for 2 or 6 days in MEM + or MEM + supplemented with different concentrations of FSH (10, 50, 100 or 200 ng/ml). [A,B]Values within a treatment (culture day – D2) with different superscripts differ significantly at $P < 0.05$ (between treatments – Control, MEM + , FSH 10, FSH 50, FSH 100 and FSH 200). [a,b]Values within a treatment (culture day – D6) with different superscripts differ significantly at $P < 0.05$ (between treatments – Control, MEM + , FSH 10, FSH 50, FSH 100 and FSH 200).

Morphometry analysis of preantral follicles cultured in vitro

For the morphometric analysis, the follicle and oocyte dimensions were measured in ovarian tissue cultured for 2 or 6 days. The mean follicular and oocyte diameters at days 2 and 6 of culture can be found in Table 1.

After culture, the mean diameter of the preantral follicles (primordial, primary and secondary) was higher at the concentration of 100 ng/ml FSH than that with the other treatments at 2 days and 6 days of culture (34.5 ± 10.8 μm and 33.2 ± 12.5, respectively; $P < 0.05$). The mean follicular diameter of primordial follicles was higher at concentrations of 100 and 200 ng/ml at 2 days of culture ($P < 0.05$), whereas at 6 days all treatments had similar alterations in morphology ($P > 0.05$).

The mean follicle diameters showed significant differences, as the concentration of 100 ng/ml FSH at 2 and 6 days led to mean diameters of 26.7 ± 5.6 and 25.3 ± 4.0 μm for primary follicles, respectively ($P < 0.05$). The mean diameters for secondary follicles did not show differences between measurements at 2 or 6 days of culture ($P > 0.05$). Regarding the diameter of the oocytes present in the follicles, at all stages of development, no significant difference was observed ($P > 0.05$; Table 1).

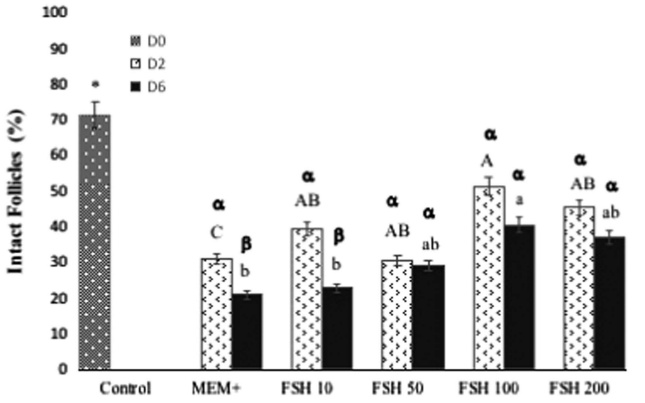

Figure 5. Percentage of intact follicles from *Bos indicus* females in early development stage (primordial, primary + secondary) in non-cultured ovarian tissue (control, day 0) and after *in vitro* culture for 2 or 6 days in MEM + or MEM + supplemented with different FSH concentrations (10, 50, 100 or 200 ng/ml). [A,B]Values within a treatment (culture day – D2) with different superscripts differ significantly at $P < 0.05$ (between treatments – Control, MEM, + FSH 10, FSH 50, FSH 100 and FSH 200). [a,b]Values within a treatment (culture day – D6) with different superscripts differ significantly at $P < 0.05$ (between treatments – Control, MEM + , FSH 10, FSH 50, FSH 100 and FSH 200). [α,β]Values within the same treatment (D2 vs. D6) with different superscripts differ significantly at $P < 0.05$ (treatments – Control, MEM + , FSH 10, FSH 50, FSH 100 and FSH 200).

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Table 1. Mean (± standard deviation, SD) of follicle/oocyte diameter in micrometres (μm) of bovine preantral follicles cultured for 2 and 6 days with MEM + or MEM + supplemented with different concentrations of FSH (10, 50, 100 and 200 ng/ml)

Treatment	Primordial	Primary	Secondary	Mean
Follicles ± standard deviation (SD) (*n*)				
MEM + D2	27.9 ± 5.2 (3)^{A,B}	24.6 ± 4.6 (18)^{A,B}	46.8 ± 19.1 (4)^{A}	28.6 ± 8.8(25)^{B,C,D}
FSH 10 D2	25.5 ± 4.6 (5)^{A,B}	21.7 ± 3.2 (19)^{B,C}	41.6 ± 0 (1)^{A}	23.2 ± 4.8(25)^{D,E}
FSH 50 D2	29.6 ± 3.8 (10)^{A,B}	22.6 ± 4.7 (13)^{A,B,C}	43.2 ± 9.3 (2)^{A}	27.1 ± 6.2(25)^{A,B,C,D}
FSH 100 D2	32.9 ± 2.4 (6)^{A}	26.7 ± 5.6 (14)^{A}	58.6 ± 16.3 (5)^{A}	34.5 ± 10.8(25)^{A}
FSH 200 D2	31.4 ± 4.2 (11)^{A}	24.7 ± 3.6 (12)^{A,B}	52.2 ± 7.9 (2)^{A}	29.8 ± 6.8(25)^{A,B}
MEM + D6	31.3 ± 4.1 (7)^{A,B}	24.3 ± 5.2 (17)^{A,B,C}	37.4 ± 0 (1)^{A}	26.8 ± 6.2(25)^{A,B,C,D}
FSH 10 D6	29.6 ± 0.9 (3)^{A,B}	20.9 ± 2.8 (21)^{C}	66.6 ± 0 (1)^{A}	23.8 ± 5.7(25)^{E}
FSH 50 D6	27.6 ± 6.3 (7)^{A,B}	24.4 ± 5.3 (18)^{A,B,C}	- (0)	25.3 ± 5.8(25)^{C,D,E}
FSH 100 D6	32.0 ± 0 (1)^{A,B}	25.3 ± 4.0 (19)^{A}	63.1 ± 19.4 (5)^{A}	33.2 ± 12.5(25)^{A,B,C}
FSH 200 D6	24.3 ± 4.0 (5)^{B}	24.3 ± 3.4 (19)^{A,B}	88.4 ± 0 (1)^{A}	26.9 ± 6.6(25)^{C,D,E}
Oocytes ± standard deviation (SD) (*n*)				
MEM + D2	7.7 ± 0.3 (3)^{a}	6.7 ± 1.0 (18)^{a}	9.0 ± 2.3 (4)^{a}	7.2 ± 1.3 (25)^{a}
FSH 10 D2	8.0 ± 1.1 (5)^{a}	6.7 ± 1.0 (19)^{a}	5.1 ± 0 (1)^{a}	6.9 ± 1.2 (25)^{a}
FSH 50 D2	7.5 ± 0.8 (10)^{a}	6.4 ± 1.2 (13)^{a}	7.3 ± 0.6 (2)^{a}	6.9 ± 1.0 (25)^{a}
FSH 100 D2	8.4 ± 1.0 (6)^{a}	6.6 ± 1.2 (14)^{a}	8.5 ± 0.9 (5)^{a}	7.4 ± 1.3 (25)^{a}
FSH 200 D2	8.6 ± 1.9 (11)^{a}	6.8 ± 0.9 (12)^{a}	7.8 ± 1.5 (2)^{a}	7.7 ± 1.5 (25)^{a}
MEM + D6	9.0 ± 1.6 (7)^{a}	6.8 ± 0.8 (17)^{a}	7.8 ± 0 (1)^{a}	7.4 ± 1.2 (25)^{a}
FSH 10 D6	8.8 ± 0.7 (3)^{a}	6.3 ± 1.2 (21)^{a}	10.3 ± 0 (1)^{a}	6.7 ± 1.4 (25)^{a}
FSH 50 D6	6.9 ± 0.7 (7)^{a}	6.9 ± 1.4 (18)^{a}	– (0)	6.9 ± 1.2 (25)^{a}
FSH 100 D6	6.5 ± 0 (1)^{a}	6.9 ± 0.8 (19)^{a}	8.4 ± 1.9 (5)^{a}	7.2 ± 1.0 (25)^{a}
FSH 200 D6	7.4 ± 1.2 (5)^{a}	6.5 ± 0.8 (19)^{a}	11.2 ± 0 (1)^{a}	6.8 ± 1.1 (25)^{a}

^{A,B}Values within a column with different superscripts differ significantly at $P < 0.05$ (between follicular diameter and treatments – MEM +, FSH 10, FSH 50, FSH 100 and FSH 200).
^{a,b}Values within a column with different superscripts differ significantly at $P < 0.05$ (between oocyte diameter and treatments – MEM +, FSH 10, FSH 50, FSH 100 and FSH 200).

Discussion

This is the first study to evaluate the effect of different concentrations of FSH on the survival and development of bovine preantral follicles enclosed in ovarian tissue cultured *in vitro in situ*. We identified positive effects of the addition of FSH on survival and growth of follicles in the initial stage of development.

The addition of 100 ng/ml FSH exhibited better conditions for follicular growth and development and maintained the viability and integrity of preantral follicles in cultivation. The effect of adding FSH observed in this study showed that the follicles present in ovarian tissue (*in situ*) of cattle for 2 and 6 days cultivation responded to FSH in a dose-dependent manner. Similarly, 100 ng/ml FSH also efficiently maintained the survival of follicle cultures isolated from goats and cross-bred cows (6 days: Barros *et al.*, 2013; 12 days: Rossetto *et al.*, 2016; respectively). However, other studies have described a lower concentration requirement (1 μm/ml and 50 ng/ml) of FSH for supplementation of the culture medium (isolated follicles: bovine – Gutierrez *et al.*, 2000 and *in situ* follicles: goats – Matos *et al.*, 2007; Magalhães *et al.*, 2009; sheep – Lima *et al.*, 2013; equine – Aguiar *et al.*, 2016). Those differences between the FSH concentration and also culture time for follicular development may be related to the differences between the species and also the methods for culture of follicles (isolated or *in situ*).

At 6 days of culture, the development of cultured follicles seemed to be non-dose dependent (10, 50, 100 and 200 ng/ml FSH). We believe that a small amount of FSH was sufficient to allow the development of the preantral follicles. Our results demonstrated that FSH is a possible substance needed in the base medium of bovine preantral follicle cultures, as it maintains cellular organization and it is responsive to the stimulatory effects of this gonadotrophin. This corroborates results described by Roy & Albee (2000), in which the presence of FSH is essential for the differentiation of somatic cells of granulosa cells during early development of primordial follicles.

The morphometric responses at 2 days of culture showed that the mean follicle growth was higher with the 100 ng/ml FSH treatment than those of the other treatments. Different results were found for follicular growth in other species, including goats (Matos *et al.*, 2007), dog (Serafim *et al.*, 2015) and equine (Aguiar *et al.*, 2016), in which treatment with 50 ng/ml FSH allowed a

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larger follicle diameter. However, at 6 days of culture, there was no significant difference in follicular diameter for any concentration of FSH.

FSH is essential for normal development of the gonads, control of steroidogenesis (Souza *et al.*, 2017) and to complete the folliculogenesis. The importance of FSH for the growth of antral follicles *in vivo* is well known. Conversely, the action of FSH in preantral follicles (primordial, primary and secondary) remains to be better understood (Adriaens *et al.*, 2004). *In vitro* studies have identified FSH as responsible for stimulation of oocyte growth and the transition from primary to secondary stage of follicles (Hulshof *et al.*, 1995). In addition, FSH also contributes to formation of the antrum, estradiol production, follicle survival and meiotic resumption (Silva *et al.*, 2016).

Moreover, the FSH concentration curve provided evidence for the role of this gonadotropin in the proliferation and differentiation of granulosa cells in primordial follicles as described by Thomas *et al.* (2005) and Duarte *et al.* (2013). Other studies have also shown the importance of FSH in the early stages of follicular development (primordial, primary and secondary), with or without growth factors (Derrar *et al.*, 2000; Cushman *et al.*, 2002; Vasconcelos *et al.*, 2013).

In this study, follicles were cultured in the ovarian tissue from cattle, which favours the interaction of follicular cells with the adjacent cells. The information about *in situ* cultivation in bovine ovary is scarce. However, research has shown the cultivation of isolated preantral follicles with FSH supplementation in various species (humans: Wright *et al.*, 1999; goats: Matos *et al.*, 2007; sheep: Barros *et al.*, 2013; swine: Wu *et al.*, 2007; canine: Serafim *et al.*, 2015 and bovine: Rossetto *et al.*, 2016).

Considering these perspectives, our study demonstrated advantages for including FSH in the *in vitro* culture of bovine follicles. The interaction of FSH with granulosa cells was observed by Saraiva *et al.* (2010) and Rossetto *et al.* (2016). In addition, this hormone exerts influence during steroidogenesis, which in turn provides the interaction of granulosa cells with theca cells (Wandji *et al.*, 1996; Thomas *et al.*, 2003). In addition to the direct participation of FSH on granulosa cells, the role of FSH in other steps of folliculogenesis has also been studied (Hsueh *et al.*, 2015).

FSH receptor expression was also performed for the transition from primary to secondary follicles and subsequently follicle (Oktay *et al.*, 1997). In the same context, the presence of FSH receptors was described in primordial follicles (Médure *et al.*, 2002). More recently, it has been shown that FSH may influence the *in vitro* differentiation and proliferation of granulosa cells (Vasconcelos *et al.*, 2013), which corroborates the results found in the present study.

Therefore, it is possible to note that for 2 days of growth, a high concentration of FSH was required (100 ng/ml) to initiate follicular activation, which is measured by the differentiation capacity of granulosa cells. This finding may suggest that in early follicular development, FSH acts at high concentrations. For 6 days of culture, lower concentrations were also effective (10, 50, 100 or 200 ng/ml), demonstrating that demand cannot exceed gonadotropin dose dependence in more advanced stages of follicular development.

The exact mechanism by which FSH acts on follicular cells is still unknown, considering viable stimulation of cell growth by indirect influence on local interactions of mesenchymal cells present in the ovarian epithelium (Nilsson & Skinner, 2001). In this context, our *in situ* culture model provides more interesting conditions than the isolated growing follicles, in which no such interaction exists between the follicular cells and ovarian stroma.

Despite consistent advances in the beneficial effects of FSH, it is important to consider the action of the other constituents in the medium that can exert synergistic action with this gonadotropin. Several substrates are used for the supplementation of culture medium, such as pyruvate, hypoxanthine, glutamine, ITS, and BSA. The medium used for the *in vitro* culture are rich in oxygen, nutrients and other elements present in the ovarian cortex (Rossetto *et al.*, 2013), and all of these components need to be considered for a correct understanding of follicular physiology.

In conclusion, the addition of FSH demonstrated better conditions for follicular growth and development and for maintaining the viability and integrity of preantral follicles in cultivation for a short period. The optimal concentration of FSH for growing preantral follicles of bovine was 100 ng/ml, which allowed for the development and maintenance of integrity during ovarian tissue in *in vitro* culture for a short period.

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