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# FSH supplementation to culture medium is beneficial for activation and survival of preantral follicles enclosed in equine ovarian tissue

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ABSTRACT

This study investigated the effect of adding different concentrations of bovine recombinant follicle-stimulating hormone on the IVC of equine preantral follicles enclosed in ovarian tissue fragments. Randomized ovarian fragments were fixed immediately (fresh non-cultured control) or cultured for 1 or 7 days in α-MEM$^+$ supplemented with 0, 10, 50, and 100 ng/mL FSH and subsequently analyzed by classical histology. Culture media collected on Day 1 or Day 7 and were analyzed for steroids (estradiol and progesterone) and reactive oxygen species (ROS). After Day 1 and Day 7 of culture, 50-ng/mL FSH treatment had a greater ($P < 0.05$) percentage of morphologically normal follicles when compared to the other groups, except the 10-ng/mL FSH treatment at Day 1 of culture. The percentage of developing follicles (transition, primary, and secondary), and follicular and oocyte diameters were higher ($P < 0.05$) in the 50-ng/mL FSH treatment compared to the other groups after Day 7 of culture. Furthermore, estradiol secretion and ROS production were maintained ($P > 0.05$) throughout the culture in the 50-ng/mL FSH treatment. In conclusion, the addition of 50 ng/mL of FSH promoted activation of primordial follicles to developing follicles, improved survival of preantral follicles, and maintained estradiol and ROS production of equine ovarian tissue after 7 days of culture.

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## 1. Introduction

Assisted reproductive biotechnologies in horses have largely been used to maximize reproductive potential in valuable or endangered horses [1,2]. However, the efficiency of assisted reproduction is hampered because of reduced recovery of fertilizable oocytes. One approach to improve reproductive efficiency is the use of IVC of ovarian preantral follicles [3].

Several substances have been shown to improve *in vitro* survival and development of preantral follicles in domestic animals, including hormones (FSH [4]; insulin [5]) and growth factors (growth differentiation factor-9 [GDF-9 [6]]; bone morphogenetic protein-15 [BMP-15 [7]]; epidermal growth factor [8]). Among these substances, the role of FSH in *in vitro* follicle culture [9–11] is remarkable.

FSH elicits effects by binding to its receptors located in primordial and primary preantral follicles in goats [12] and sheep [13], and in horses, in primordial, primary,

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secondary, and tertiary follicles and in CL [14]. These findings suggest that FSH seems to be important even during early folliculogenesis (preantral follicular phase). Reports have shown that FSH added to culture medium maintains follicle viability and promotes the development of isolated preantral follicles and antrum formation in a concentration-dependent manner in different species (primate: [15]; bovine: [16]; human: [17]; caprine: [18]; and ovine: [19]). Furthermore, FSH can act indirectly by stimulating the expression of kit-ligand, GDF-9, and BMP-15 [20,21], which play important roles in folliculogenesis.

IVC studies of preantral follicles enclosed in ovarian tissue fragments (*in situ* culture) have shown that the ideal concentration of FSH to be added in culture to improve follicle survival and development varies according to species (50 ng/mL, caprine: [22,23]; 100 ng/mL, ovine: [24]; 100 ng/mL, canine: [25]; 50 ng/mL, bovine: [26]; and 25 mIU/mL, murine: [27]). However, despite the importance of FSH on early folliculogenesis in many species, the impact of FSH on *in vitro* development of equine preantral follicles enclosed in ovarian tissue remains unknown.

Therefore, the objective of this study was to evaluate the effect of different concentrations of FSH (0, 10, 50, and 100 ng/mL) on the survival, activation, hormone production (estradiol and progesterone), and reactive oxygen species (ROS) generation after IVC of equine preantral follicles enclosed in ovarian tissue fragments.

## 2. Materials and methods

### 2.1. Chemicals

Unless otherwise noted, the culture media and other chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, Mo, USA).

### 2.2. Animals and ovaries

This research protocol (#12637266-7) was approved by the Ethics and Animal Use Committee of the State University of Ceará, Fortaleza, CE. The ovaries were harvested from euthanized mares (n = 5) positive but asymptomatic for Equine Infectious Anemia, during September to January, in the state of Ceará, Brazil. The mares were crossbred, 6 to 10-years old (mean, 7.2 ± 1.9 years), and had body condition scores between 4 and 6 (1, emaciated to 9, obese [28]). Dental characteristics [29] were used to estimate the age of the mares. At the time of ovary harvesting, mares were nonpregnant and cycling, on the basis of presence of large (maturing/growing) or small (regressing) CL and preovulatory (>30 mm) follicle in one or both ovaries.

Immediately after euthanasia, the ovaries were collected and washed in 70% alcohol, followed by two washes in minimum essential medium (MEM) supplemented with 25-mM HEPES. The ovaries were placed into tubes containing 100 mL of MEM supplemented with 100-μg/mL penicillin and 100-μg/mL streptomycin and transported to the laboratory at 4 °C within 4 hours [30]. In the laboratory, the ovaries of each animal were stripped of surrounding fat tissue and ligaments. Subsequently, 72 ovarian tissue samples (approximate size, 3 × 3 × 1 mm) were obtained from each pair of ovaries under sterile conditions using a scalpel blade. Eight ovarian tissues samples were distributed for each treatment per day in five replicates.

### 2.3. Culture of preantral follicles and experimental design

Ovarian tissue was placed in 24-well culture plate containing 1 mL of culture media. Culture was performed at 39 °C in a humidified atmosphere with 5% $CO_2$ in air. Fresh medium was prepared and incubated for at least 1 hour before use. The basic culture medium consisted of α-MEM (pH 7.2–7.4) supplemented with 2-mM glutamine, 2-mM hypoxanthine, 1.25-mg/mL BSA, 10-ng/mL insulin, 5.5-μg/mL transferrin, 5-ng/mL selenium, 100-μg/mL penicillin, and 100-μg/mL streptomycin, which is called α-MEM$^+$.

To test the effect of FSH in the culture of preantral follicles, the basic medium (control) was supplemented with increasing concentrations of bovine recombinant FSH (rbFSH; Nanocore, Campinas, SP, Brazil) generating the following treatments: 0 ng/mL, 10 ng/mL, 50 ng/mL, and 100 ng/mL. The ovarian fragment pieces were then either fixed for histologic analyses (fresh, noncultured control group) or placed in culture for 1 or 7 days. Five replicates were performed for each treatment. The culture media were replaced every other day, and before each replacement, 1 mL of medium was collected at Day 1 and Day 7 of culture and stored at −80 °C for hormonal and ROS analyses.

### 2.4. Morphologic evaluation and follicle development

Follicular morphology and development of preantral follicles were assessed *in situ* (ovarian fragments) before and after IVC of ovarian tissue. Once harvested, ovarian tissue submitted to histologic analysis was fixed in paraformaldehyde solution at 4 °C for 12 hours and kept in 70% alcohol. Ovarian fragments were dehydrated by use of a graded series of alcohol, embedded in paraffin wax, and cut into serial sections at 10 μm. Samples were stained with periodic acid-Schiff and counterstained with hematoxylin. Histology slides were analyzed using light microscopy (Nikon, Tokyo, Japan) at magnification × 400. The histologic evaluation and classification of the follicles were performed by only one operator who was blind to the treatments, although other five trained helpers, also blind to the treatments, have been used to find preantral follicles on the histologic sections of each fragment.

The end points evaluated were number of follicles, follicle class (primordial, one layer of flattened pregranulosa cells surrounding the oocyte; transitional, one layer of flattened and cuboidal granulosa cells; primary, one layer of cuboidal granulosa cells; and secondary, two or more layers of cuboidal granulosa cells; [17]), follicle morphology, and diameter of follicles and oocytes.

To evaluate follicular development, the percentage of both normal primordial and developing follicles (transitional, primary, and secondary; Fig. 1) was calculated before (fresh noncultured control group) and after culture for each treatment. Regarding morphology, follicles were classified as normal (follicle containing an intact oocyte and

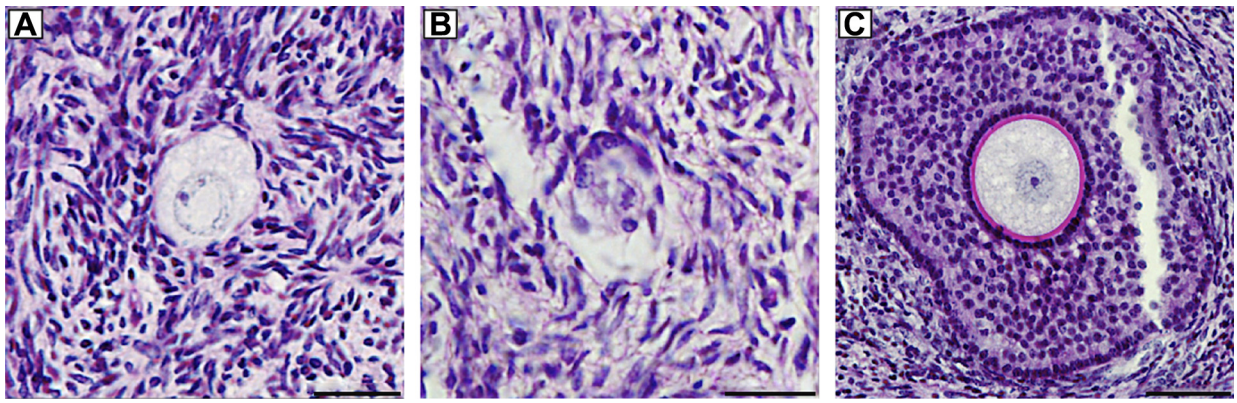


**Fig. 1.** Morphologic aspects of preantral follicles after 7 days of culture in 50-ng/mL FSH treatment. (A) normal primordial follicle, (B) abnormal transitional follicle, and (C) secondary normal follicle. Bars = 25 μm (A, B) and 50 μm (C).

granulosa cells well organized in layers without pyknotic nucleus) or abnormal (oocyte with pyknotic nucleus, retracted cytoplasm, or disorganized granulosa cells detached from the basement membrane). Every follicle was examined in each section in which it appeared and matched with the same follicle on adjacent sections to avoid double counting, thereby ensuring that each follicle was counted only once.

Follicle and oocyte diameters were measured only in morphologically normal follicles using software (Nis-Element AR 3.0) coupled with an epifluorescence microscope (Zeiss, Cologne, Germany). Follicle diameter was measured from one edge to the other edge of the outermost layer of granulosa cells. Oocyte diameter was measured from one edge of the oocyte membrane to the other. Two perpendicular diameters were recorded for each measurement, and the average of those two values was calculated.

### 2.5. Hormone analyses

To evaluate follicular steroidogenesis *in vitro*, concentrations of estradiol and progesterone were measured in reserved culture media against standard dilutions, according to manufacturer's instructions, using competitive immunoassay commercial kits: IMMULITE 2000 Estradiol (catalog No: L2KE22 Siemens Medical Solutions Diagnostics, 5210 Pacific Concourse Drive, Los Angeles, CA, USA) and IMMULITE 2000 Progesterone (catalog No: L2KPW2 Siemens Medical Solutions diagnostics, 5210 Pacific Concourse Drive, Los Angeles, CA, USA). The two assays have been previously used and validated for different species, including the horse [31,32]. The analytical sensitivity of the assay was 15 pg/mL (assay range, 20–2000 pg/mL) for estradiol and 0.1 ng/mL (assay range, 0.2–40 ng/mL) for progesterone.

### 2.6. ROS analysis

The levels of ROS were determined in reserved culture media using a spectrofluorimetric method [33]. Culture media from all FSH-treated groups were incubated with 10 μl of 2', 7'-dichlorofluorescin diacetate (DCHF-DA; 1 mM). The oxidation of DCHF-DA to dichlorofluorescein was measured for detection of reactive species in the medium. The intensity of fluorescence emission was recorded at 520 nm (with 480 nm excitation) for 2 hours after addition of the DCHF-DA to the medium.

### 2.7. Statistical analyses

Follicular and oocyte diameter end points were not normally distributed according to a Kolmogorov–Smirnov test and were transformed to natural logarithms and ranks, respectively. Diameters of preantral follicles and oocytes, and concentrations of hormones and ROS were analyzed to determine the main effects of treatment groups, day, and their interaction. One-way ANOVA and SAS PROC MIXED procedure were used (9.3 version; SAS Institute Inc., Cary, NC, USA). If a significant effect of treatment or treatment-by-day interaction was detected, a Duncan test for multiple comparisons was used to discern differences in means among groups. If a significant day effect was obtained, differences between means within a group were examined by unpaired Student's *t* tests. Chi-square analysis was used to compare the percentage of morphologically normal and growing preantral follicles among groups within each day and between days within each group. A probability of $P \leq 0.05$ indicated that a difference was significant, and probabilities between $P > 0.05$ and $\leq 0.1$ indicated that a difference approached significance. Data are presented as the mean ± standard error of the mean, unless otherwise indicated.

## 3. Results

### 3.1. Number of fragments and follicles evaluated

A total of 3600 slides with 36,000 tissue sections were produced from 360 ovarian fragments. At least 30 follicles (range, 30–54 follicles) were evaluated per treatment and per day in each replicate, allowing each animal to contribute in a similar way to the FSH treatments. A total of 206, 337, 373, 345, and 342 preantral follicles were

evaluated on the fresh noncultured control, 0-ng/mL FSH, 10-ng/mL FSH, 50-ng/mL FSH, and 100-ng/mL FSH treatment groups, respectively. Altogether, 1603 follicles were evaluated with an average of 320.6 ± 29.3 follicles evaluated per treatment.

### 3.2. Effect of FSH on follicular morphology

The percentage of morphologically normal follicles was reduced after Day 1 and Day 7 of culture ($P < 0.01$) when compared to fresh noncultured control (Table 1). Moreover, after Day 1 and Day 7 of culture, the 50-ng/mL FSH treatment had a higher ($P < 0.05$) percentage of normal follicles than the other treatments, except for the 10-ng/mL FSH at Day 1 of culture. After Day 7 of culture, FSH 50 ng/mL tended ($P < 0.07$) to differ from the 10-ng/mL treatment. A reduction ($P < 0.003$) in the percentage of normal follicles was observed in all treatments from Day 1 to Day 7 of culture.

### 3.3. Follicular development after IVC

The percentages of primordial and developing follicles are shown (Fig. 2). After Day 1 of culture, the 10-ng/mL FSH and 50-ng/mL FSH treatments decreased ($P < 0.02$) the percentage of primordial follicles and increased the percentage of growing follicles when compared to the fresh noncultured control group. In all treatments, after Day 7 of culture, the percentage of primordial follicles decreased ($P < 0.02$) and the percentage of growing follicles increased ($P < 0.02$) when compared to fresh noncultured control. The 50-ng/mL FSH treatment had the highest ($P < 0.05$) rate of growing follicles on Day 7 of culture among all treatments. All treatments had a decrease ($P < 0.005$) in percentage of primordial follicles and an increase in developing follicles during IVC.

After Day 1 of culture, the 50-ng/mL FSH was the treatment that maintained follicular and oocyte diameters similar ($P > 0.05$) to fresh noncultured control group. In addition, the 50-ng/mL FSH treatment showed greater ($P < 0.0001$) follicular and oocyte diameter than the other FSH treatments, except for the oocyte diameter in the 0-ng/mL FSH treatment (Tables 2 and 3). However, after Day 7 of culture, all treatments had lower ($P < 0.0001$) follicular and oocyte diameters when compared to the fresh noncultured control. After Day 7 of culture, the 50-ng/mL FSH treatment had greater ($P < 0.05$) follicular and oocyte diameters than all other FSH treatments. Regardless of treatment, follicular and oocyte diameters decreased ($P < 0.03$) from Day 1 to Day 7 of culture.

**Table 1**
Percentage of morphologically normal equine preantral follicles in fresh noncultured ovarian tissue fragments and after IVC for 1 or 7 days in media supplemented with different concentrations of bovine recombinant follicle-stimulating hormone.

| | Percentage of normal follicles (n) | |
|---|---|---|
| Fresh noncultured control | 76.2 (157/206) | |
| FSH treatments | Day 1 | Day 7 |
| 0 ng/mL | 52.2 (94/180)[c,A,C,a] | 31.2 (49/157)[c,A,b] |
| 10 ng/mL | 59.1 (110/186)[c,A,B,a] | 32.6 (61/187)[c,A,b] |
| 50 ng/mL | 65.8 (100/152)[c,B,a] | 41.4 (80/193)[c,B,d,b] |
| 100 ng/mL | 45.1 (78/173)[c,C,a] | 29.6 (50/169)[c,A,b] |

[A,B,C]Within days, uncommon uppercase superscripts differed ($P < 0.05$–0.008).
[a,b]Within treatments, uncommon lowercase superscripts differed ($P < 0.003$–0.0001).
[c] Values differed ($P < 0.01$) from the fresh noncultured control.
[d] Tended ($P < 0.07$) to differ from the 10 ng/mL treatment on Day 7.

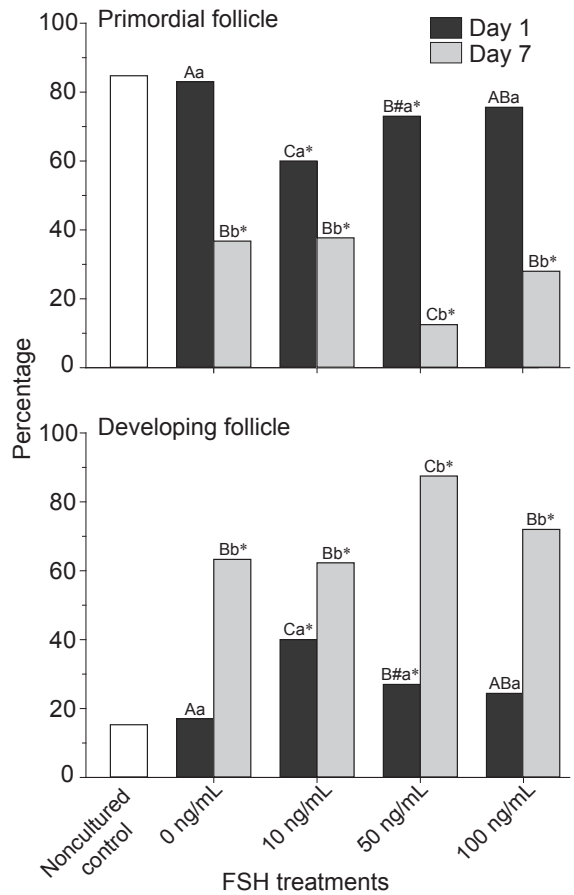


**Fig. 2.** Percentage of primordial and developing follicles (transitional, primary, and secondary; n = 779) in fresh noncultured ovarian tissue fragments and after IVC for 1 or 7 days in media supplemented with different concentrations of bovine recombinant follicle-stimulating hormone (mean, 86.5 follicles/treatment/day). [a,b]Within each treatment, values without a common letter differed ($P < 0.005$). [A,B,C]Within days, values without a common letter differed ($P < 0.05$). [#]Tended ($P < 0.09$) to differ from 0-ng/mL FSH treatment at Day 1. [*]Differed ($P < 0.02$) from fresh noncultured control.

### 3.4. Estradiol and progesterone analyses

Estradiol production was not different among treatments ($P > 0.05$; Fig. 3). However, estradiol concentration decreased ($P < 0.0001$) in all FSH treatments from Day 1 to Day 7 of culture, except for the 50-ng/mL treatment. Progesterone production in the culture media was detected in a small number of samples (19 out of 160; data not shown). Therefore, data were not analyzed statistically.

**Table 2**
Mean (±standard error of the mean) diameters of equine preantral follicles (primordial, transitional, and primary combined) in fresh noncultured ovarian tissue fragments and after IVC for 1 or 7 days in media supplemented with different concentrations of bovine recombinant follicle-stimulating hormone.

| | Diameter (μm) | |
|---|---|---|
| Fresh noncultured control | 38.5 ± 0.3 | |
| FSH treatments | Day 1 | Day 7 |
| 0 ng/mL | 35.1 ± 0.6[c,B,a] | 28.8 ± 0.6[c,B,b] |
| 10 ng/mL | 34.8 ± 0.8[c,B,a] | 30.9 ± 0.7[c,B,b] |
| 50 ng/mL | 39.1 ± 1.3[A,a] | 35.8 ± 1.0[c,A,b] |
| 100 ng/mL | 33.0 ± 0.8[c,B,a] | 30.1 ± 0.6[c,B,b] |

[A,B]Within days, uncommon uppercase superscripts differed ($P < 0.0001$).
[a,b]Within treatments, uncommon lowercase superscripts differed ($P < 0.03$–$0.0001$).
[c] Values differed ($P < 0.0001$) from the fresh noncultured control.

### 3.5. ROS production

After Day 1 of culture, no differences ($P > 0.05$) among treatments were observed in ROS production. However, after Day 7 of culture, the 50-ng/mL FSH treatment produced more ($P < 0.05$) ROS only when compared to the 0-ng/mL FSH treatment (Table 4). The concentrations of ROS decreased from Day 1 to Day 7 of culture in the 0-ng/mL ($P < 0.07$) and in the 10-ng/mL ($P < 0.05$) FSH treatments. On the other hand, the 50-ng/mL and 100-ng/mL FSH treatments maintained ROS levels during the culture time.

## 4. Discussion

This study demonstrated for the first time that adding FSH in a concentration-dependent manner improved both *in vitro* follicular survival and activation of equine primordial follicles enclosed in ovarian tissue.

The addition of 50-ng/mL FSH was able to provide a greater percentage of morphologically normal follicles than all tested concentrations after Day 7 of culture. In goats, Magalhães et al. [23] used a concentration-response curve of bovine recombinant follicle-stimulating hormone (0, 10, 50, 100, and 1000 ng/mL), and reported that 50 ng/mL maintained survival and follicular ultrastructure, and promoted the activation and growth of primordial follicles after Day 7 of culture. FSH receptors have been reported to be expressed in oocytes of primordial follicles of porcine and primary follicles in humans [34] and in granulosa cells from the primary follicle stage onward in horses [14]. The positive effect of FSH observed on *in vitro* follicle culture in the present study might have been because of its direct and/or indirect action. Among the direct effects is the activation of genes that code for the stimulation of cell proliferation and steroid synthesis [35]. Indirectly, FSH regulates the expression of some of the many important substances that play a role on folliculogenesis, such as kit-ligand, GDF-9, and BMP-15 [20].

The addition of 50-ng/mL FSH to the culture medium reduced the proportion of primordial follicles and caused a

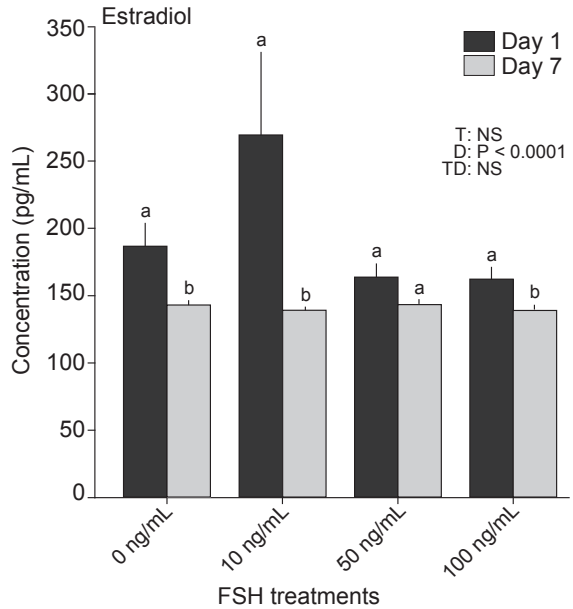


**Fig. 3.** Mean (±standard error of the mean) concentrations of estradiol (pg/mL; n = 160 samples) produced by equine ovarian tissue fragments after IVC for 1 or 7 days in media supplemented with different concentrations of bovine recombinant follicle-stimulating hormone (mean, 20 samples/treatment/day). [a,b]Within each treatment, values without a common letter differed ($P < 0.0001$). No difference within days was observed among treatments. T, treatment; D, day; TD, treatment × day.

**Table 3**
Mean (±standard error of the mean) diameters of equine oocytes from preantral follicles (primordial, transitional, and primary combined) in fresh noncultured ovarian tissue fragments and after IVC for 1 or 7 days in media supplemented with different concentrations of bovine recombinant follicle-stimulating hormone.

| | Diameter (μm) | |
|---|---|---|
| Fresh noncultured control | 30.6 ± 0.4 | |
| FSH treatments | Day 1 | Day 7 |
| 0 ng/mL | 28.8 ± 0.5[A,a] | 22.1 ± 0.6[c,B,b] |
| 10 ng/mL | 26.7 ± 0.7[c,B,a] | 23.9 ± 0.7[c,B,b] |
| 50 ng/mL | 30.9 ± 1.0[A,a] | 27.9 ± 0.9[c,A,b] |
| 100 ng/mL | 26.3 ± 0.7[c,B,a] | 23.3 ± 0.5[c,B,b] |

[A,B]Within days, uncommon uppercase superscripts differed ($P < 0.0001$).
[a,b]Within treatments, uncommon lowercase superscripts differed ($P < 0.03$–$0.0001$).
[c] Values differed ($P < 0.0001$) from the fresh noncultured control.

**Table 4**
Mean (±standard error of the mean) reactive oxygen species (relative fluorescence units) produced by equine ovarian tissue fragments after IVC for 1 or 7 days in media supplemented with different concentrations of bovine recombinant follicle-stimulating hormone.

| | Relative fluorescence units | |
|---|---|---|
| FSH treatments | Day 1 | Day 7 |
| 0 ng/mL | 32.4 ± 2.3[A] | 26.9 ± 1.3[A,c] |
| 10 ng/mL | 34.6 ± 2.0[A,a] | 28.0 ± 1.3[A,B,b] |
| 50 ng/mL | 30.8 ± 2.1[A,a] | 31.1 ± 1.6[B,a] |
| 100 ng/mL | 29.2 ± 2.3[A,a] | 30.6 ± 1.9[A,B,a] |

[A,B]Within days, uncommon uppercase superscripts differed ($P < 0.05$).
[a,b]Within treatments, uncommon lowercase superscripts differed ($P < 0.05$).
[c] ROS production tended ($P < 0.07$) to differ between Days 1 and 7.

concomitant increase in the proportion of growing follicles at Day 7 of culture, indicating that primordial follicle activation had occurred. Such an effect is possibly because of the stimulatory effect of FSH on the expression of genes involved in proliferation and differentiation of granulosa cells [36], which in turn induce multiple signaling cascades [37], and can quickly stimulate the activation of mitogen-activated protein kinases pathways and phosphatidylinositol 3-kinase, which impact cell proliferation [38].

Follicular and oocyte diameters were greater after Day 7 of culture in the 50-ng/mL FSH treatment when compared to all other treatments. Similar results have been found for goat [22], and dog [25] preantral follicles. Compared with the fresh noncultured control, FSH treatment in this study was not efficient in promoting follicular growth, which is in agreement with findings using horse preantral follicle culture without supplemental FSH [39]. In addition, a possible explanation for the decrease in follicle and oocyte diameters from Day 1 to Day 7 of culture was that primary and secondary follicles are more sensitive to degeneration than primordial follicles [40]. This could explain why only few primary and secondary follicles contributed to the mean follicle and oocyte diameters in this study. Thus, studies aimed toward developing a culture system for equine preantral follicles that promotes continuous follicular and oocyte growth to late stages of folliculogenesis are warranted.

This study showed that fragments of equine ovarian tissue containing preantral follicles, when cultured *in vitro*, exhibited steroidogenic activity. Irrespective of culture time, estradiol and progesterone production were not affected by FSH concentrations. However, after Day 7 of culture, although the 50-ng/ml FSH treatment maintained estradiol levels, all other FSH treatments significantly reduced estradiol concentration. This finding might be explained by the fact that larger follicles, which have a higher steroidogenic activity, degenerate before smaller follicles [40], which have a lower steroidogenic capacity [41]. In addition, we cannot rule out that other cell types such as ovarian stromal cells could contribute to estradiol production under our culture conditions, as it has been reported that these cells are capable of producing steroids [42,43].

In this study, 50 and 100-ng/mL FSH treatments maintained ROS production between Day 1 and Day 7 of culture. However, the 50-ng/mL FSH treatment was the only one that differed (significant higher levels) from the 0-ng/mL FSH treatment (FSH control group). The evaluation of ROS production has been an important parameter used to determine the presence of free radicals that might be deleterious to cells in culture [44,45]. Also, strong evidence demonstrates the toxicity role of ROS caused by several chemical and physical agents, in the initiation of apoptosis mainly on antral follicles, resulting in poor oocyte quality and possibly having noxious effects even in early preantral follicles [46]. Therefore, FSH was added to the culture media because of its important role in protecting follicles from apoptosis by increasing glutathione levels and suppressing ROS production as described previously [47]. Furthermore, appropriate levels of ROS are extremely important for the maintenance of cellular homeostasis to modulate physiological events (e.g., ovulation), possibly by its action in activation of the phosphokinase A signaling [48] or cell growth and differentiation [49]. To date, it has been shown that FSH stimulates catalase activity in goat granulosa cells modulating intracellular ROS levels [50]. ROS inhibitors, in a concentration-dependent manner, decreased oocyte maturation induced by FSH [51]. In our study, we suggest that the presence of antioxidants (e.g., transferrin [52]; selenium [53]), insulin in physiological concentration (10 ng/mL [54]), along with an adequate FSH concentration (50 ng/mL), contributed to the maintenance of suitable levels of ROS after Day 7 of culture, resulting in higher rates of follicle survival and activation.

In conclusion, the addition of 50-ng/mL FSH promoted activation of primordial follicles to developing follicles, improved survival of preantral follicles, and maintained estradiol and ROS production of equine ovarian tissue after 7 days of culture and can be recommended for IVC of equine preantral follicles enclosed in ovarian tissue fragments. The novel findings of this study open the prospect for the use of FSH in the base culture medium and also to investigate the efficacy of other substances (such as growth factors) added to culture media to optimize equine *in vitro* follicular development.

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