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Influence of FSH on *in vitro* growth, steroidogenesis and DNA synthesis of buffalo (*Bubalus bubalis*) ovarian preantral follicles

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Abstract

The present study was designed to examine the effect of different doses of FSH on the growth, steroidogenesis and DNA synthesis of two different sizes of buffalo ovarian preantral follicles (PFs) during *in vitro* culture. Buffalo ovaries were collected from a local abattoir and PFs of small (<150 µm) and large (>150 µm) sizes were isolated through the microdissection method and cultured in TCM-199 supplemented with 10% FBS, 1% ITS and 30 ng/ml EGF (control group) at 38.5°C in 5% CO_2 with maximum humidity. The addition of three different doses of FSH (0.5, 1.0 and 2.0 µg/ml) in the control medium served as the treatment groups. The culture medium was replenished every third day and spent culture medium was stored at -30°C for steroid hormone analysis. The results showed that the supplementation of FSH in the culture medium stimulated ($P < 0.05$) follicular growth, steroidogenesis and DNA synthesis compared to the control. The addition of FSH at 1.0 µg/ml in the culture medium stimulated better ($P < 0.05$) follicular growth, DNA synthesis and steroidogenesis compared to 0.5 µg/ml FSH, whereas a higher dose of FSH (2.0 µg/ml) inhibited follicular growth in both sizes of follicles. In conclusion, the present study demonstrated that FSH plays a key role during the development of buffalo ovarian preantral follicles, as it stimulated *in vitro* survival, growth rate, steroidogenesis and DNA synthesis of buffalo preantral follicles at an optimal dose of 1.0 µg/ml.

Keywords: buffalo, DNA synthesis, FSH, preantral follicles, steroidogenesis.

Introduction

The development of culture systems to promote the growth of preantral follicles (PFs) up to the Graafian follicle stage and to acquire meiotically competent oocytes would certainly be useful to reduce generation interval, conserve genetic resources and understand folliculogenesis. Several *in vivo* and *in vitro* studies have shown that FSH facilitated the development of follicles at preantral stages in humans (Roy and Treacy, 1993), cows (Wandji *et al*., 1996; Gutierrez *et al*., 2000), goats (Matos *et al.*, 2007) and pigs (Mao *et al.*, 2002). Sudo *et al.* (2007) reported that the *in vitro* growth and survival of bovine PFs depend on the presence of physiological concentrations of gonadotropins. DNA synthesis in sheep preantral follicular cells is also induced by FSH, as evidenced by their ability to incorporate bromodeoxyuridine (BrdU; Hemamalini *et al*., 2003). The maturation of oocytes from *in vitro* cultured PFs up to the metaphase II stage was achieved with an increase in DNA synthesis in sheep preantral follicles when cultured in a medium containing gonadotropins and growth factors (Hemamalini *et al*., 2003; Tamilmani *et al.*, 2005). The incubation of intact follicles with FSH and subsequent measurement of progesterone and estradiol in the spent culture medium after the culture period can establish the functional changes in the *in vitro* cultured PFs (Kishi and Greenwald, 1999). Several culture systems have been developed with FSH facilitating the induction and maintenance of estradiol secretion in mouse (Nayudu and Osborn, 1992), sheep (Campbell *et al*., 1996) and cow granulosa cells (Gutierrez *et al*., 1997).

The role of FSH in domestic ruminants during the development of large and small preantral follicles is debatable. Webb and Armstrong (1998) showed that preantral follicles could grow up to antral follicles (2 to 4 mm in diameter) under *in vitro* culture conditions either in the absence or presence of very low concentrations of gonadotropins. Similar studies were also reported in mice (Nayudu and Osborn, 1992), rats (Camp *et al.*, 1991) and sheep (Campbell *et al.*, 1995) which further strengthened the general assumption that PFs are not gonadotropin dependent, while other studies in mice (Edward *et al.*, 1977), goats (Rossetto *et al.*, 2009; Lima-Verde *et al.*, 2010) cats (Saint-Dizer *et al.*, 2007), sheep (Dufour *et al.*, 1979) and cattle (Evans and Fortune, 1997) clearly demonstrated that the development of PFs are gonadotropin dependent and that FSH supplementation is essential to maintain follicular growth and integrity. Saraiva *et al.* (2011) reported that the addition of increased concentrations of FSH (sequential medium) had a significant impact on the *in vitro* development of caprine preantral follicles. Previously, our laboratory also demonstrated the localization of FSH receptors (Taru Sharma *et al.*, 2011) and the expression of FSH receptor genes in small and

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large buffalo PFs (Kurvila *et al*., 2010). However, an appropriate concentration of FSH, time and stage of PFs during *in vitro* development in buffalo needs to be known, along with the impact of FSH on DNA synthesis. Therefore, this study was designed to investigate the minimal effective dose of FSH in order to obtain an ideal combination of follicle growth, survival and steroidogenesis in *in vitro* developing preantral follicles.

Materials and Methods

Chemicals

All chemicals used in this study were purchased from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise indicated.

Ovaries

Buffalo ovaries from random stages of the estrous cycle were collected from a local abattoir immediately after slaughter and transported to the laboratory within 2 h. During transit, they were maintained at 25-30°C in 0.9% normal saline supplemented with antibiotics. In the laboratory, the ovaries were freed from ligaments and rinsed well in pre-warmed phosphate-buffered saline (PBS) supplemented with antibiotics (75 mg/l penicillin-G, 50 mg/l streptomycin sulfate).

Isolation and selection of PFs

Under sterile conditions, fine cortical slices (~0.5-1.0 mm) were cut from the ovarian surface using a surgical blade and placed in the follicle collection medium supplemented with sodium pyruvate (2 mM), glutamine (2 mM), bovine serum albumin (BSA, 3 mg/ml) and antibiotics (75 mg/l penicillin-G, 50 mg/l streptomycin sulfate) at room temperature. PFs (100-300 µm diameter, 20-25 follicles/experimental day) were isolated by the microdissection method as described by Taru Sharma *et al.* (2009). Healthy PFs selected for *in vitro* culture were characterized based on granulosa cell layers and a visible, centrally located oocyte. Based on their diameter and layer(s) of granulosa cells, selected follicles were classified as small (<150 µm in diameter, one layer of flat granulosa cells) and large (150-300 µm in diameter, two to three layers of granulosa cells) PFs.

In vitro culture of PFs

The basic culture medium was HEPES buffered (5 mM) tissue culture medium-199 (TCM-199) supplemented with sodium bicarbonate (26 mM). All the selected PFs were cultured with or without FSH to determine the most suitable medium. The control medium consisted of the basic culture medium supplemented with 10% fetal bovine serum (FBS), 1% ITS liquid medium (1 mg/ml insulin, 0.55 mg/ml transferrin and 0.5 µg/ml sodium selenite) and epidermal growth factor (30 ng/ml EGF). The treatments consisted of adding 0.5, 1.0 or 2.0 µg/ml FSH (Sigma, human pituitary, recombinant) in the control medium. Selected PFs of both sizes were placed in 70 µl droplets of the respective culture medium (4-5 PFs/group) in a 35 mm plastic tissue culture dish (Nunc, Denmark) and overlaid with 2 ml of embryo tested lightweight mineral oil. These culture dishes were placed in a humidified incubation chamber (Thermo Forma, USA) at 38 ± 1°C in a maximum humidified atmosphere having 5% CO_2 in air for 10 days. Half of the medium was replenished by an equal volume of fresh medium every third day. The replaced medium was stored at -30°C for steroid analysis.

Morphological evaluation of in vitro cultured PFs

At the end of the culture period, PFs from all the above mentioned treatments were collected to evaluate survival, growth, DNA synthesis and steroid production (estradiol, progesterone). The *in vitro* growth of PFs was monitored by measurement of PF diameter using Image J 1.33U software (National Institutes of Health, Bethesda, MD, USA) based on a calibrated ocular micrometer at 0 and 10 days of culture. As eccentric movement of the oocyte within the PFs is associated with growth and antrum formation, this was considered to be an indicator of *in vitro* development of PFs through examination under an inverted microscope (Olympus CKX 41, Japan). The viability of *in vitro* grown PFs was evaluated using the 0.05% (w/v) trypan blue (Sigma) dye exclusion test for 5 to 10 min. Follicles exhibiting a dark blue stain were considered as dead and were discarded.

Measurement of total DNA

The total DNA content of individual freshly isolated (day 0) and *in vitro* cultured follicles (day 10) in different treatment groups was measured. Total DNA from the PFs was isolated using the QIAamp DNA micro kit (Qiagen Inc., Germany) as per the manufacturer's protocol and quantified (ng/µl) directly using a Nano-drop 1000 UV visible spectrophotometer (Thermo Scientific, USA).

Measurement of steroids

Estradiol and progesterone were estimated in the spent culture medium collected on days 2, 4, 6 and 8 of culture using commercially available radio-immunoassay kits (Immunotech, Czech Republic). Assays for steroids were carried out as per the manufacturer's protocol using duplicate samples. The

analytical sensitivities of the progesterone and estradiol kits were 0.02 pg/ml and 4.5 pg/ml, respectively. Inter and intra assay variation coefficients for progesterone were 8.5 and 4.5% and for estradiol were 11.2 and 12.1%, respectively.

Statistical analyses

Data collected from 18 to 20 replicates on different days of culture were expressed as mean ± standard error of the mean (SEM). Statistical significance was determined using SPSS software for Windows, version 7.5 (SPSS GmbH Software, Munich, Germany) by analysis of variance (ANOVA) followed by Duncan's post-hoc multiple comparison test for proportion. A probability of $P < 0.05$ was considered to be statistically significant. Progesterone and estradiol levels were subjected to statistical analysis over the culture period at 2, 4, 6 and 8 days. These data were analyzed by ANOVA and treatments were further compared by Duncan's multiple range test.

Results

Influence of FSH on growth of small (<150 µm) and large (150-300 µm) sizes buffalo PFs

Supplementation of the culture medium with FSH at different concentrations influenced the growth, survival and maintenance of the architecture of PFs compared to the control. The addition of FSH (1.0 µg/ml) in the control medium significantly increased the growth, survivability and antrum formation of small and large PFs compared to other doses of FSH on day 10 of culture (Fig. 1). On day 10 of culture in the 1.0 µg/ml FSH group, an increase in diameter up to 158.4 and 234.1% was noticed for small and large PFs, respectively (Tables 1 and 2). At the end of culture period, nearly 70% of follicles survived from both the groups as confirmed by the trypan blue dye test. Unstained follicles were classified as viable and fully stained follicles as dead (Fig. 2).

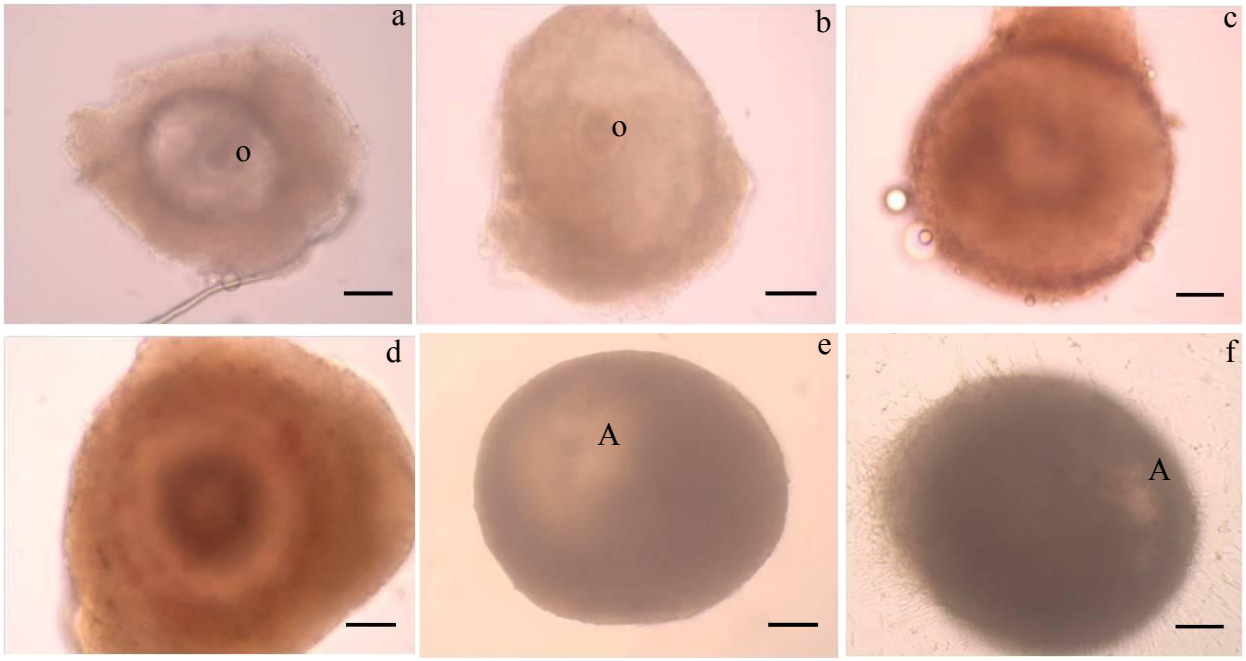

Figure 1. *In vitro* growth and development of buffalo preantral follicles: (a & b) small and large preantral follicle (day 0) showing centrally located oocyte (o), respectively, (c & d) growth of follicle during culture, (e & f) antrum formation (A) in small and large cultured preantral follicles after 10 days of culture, respectively (original magnification of photomicrographs was 200X).

Table 1. Influence of different concentrations of FSH on *in vitro* growth of small buffalo preantral follicles.

Treatment	Follicle size on Day 0 (µm) Mean ± SEM	Follicle size on Day 10 (µm) Mean ± SEM	Total increase in diameter (%)
Control	119.7 ± 2.5	156.4 ± 5.1[a]	36.7[a]
FSH 0.5 µg/ml	119.6 ± 2.7	250.1 ± 7.6[b]	130.5[b]
FSH 1.0 µg/ml	117.9 ± 2.7	276.3 ± 10.2[b]	158.4[b]
FSH 2.0 µg/ml	118.5 ± 2.5	208.7 ± 4.2[c]	90.2[c]

[a,b,c]Within columns, means with different superscripts were significantly different ($P < 0.05$).

Table 2. Influence of different concentrations of FSH on *in vitro* growth of large buffalo preantral follicles.

Treatment	Follicle size on Day 0 (µm) Mean ± SEM	Follicle size on Day 10 (µm) Mean ± SEM	Total increase in diameter (%)
Control	244.5 ± 5.3	316.3 ± 7.8[a]	71.8[a]
FSH 0.5 µg/ml	244.7 ± 6.5	428.8 ± 11.8[b]	184.1[b]
FSH 1.0 µg/ml	237.9 ± 6.4	472.0 ± 16.2[b]	234.1[b]
FSH 2.0 µg/ml	237.0 ± 5.3	329.5 ± 10.5[a]	92.5[a]

[a,b]Within columns, means with different superscripts were significantly different (P < 0.05).

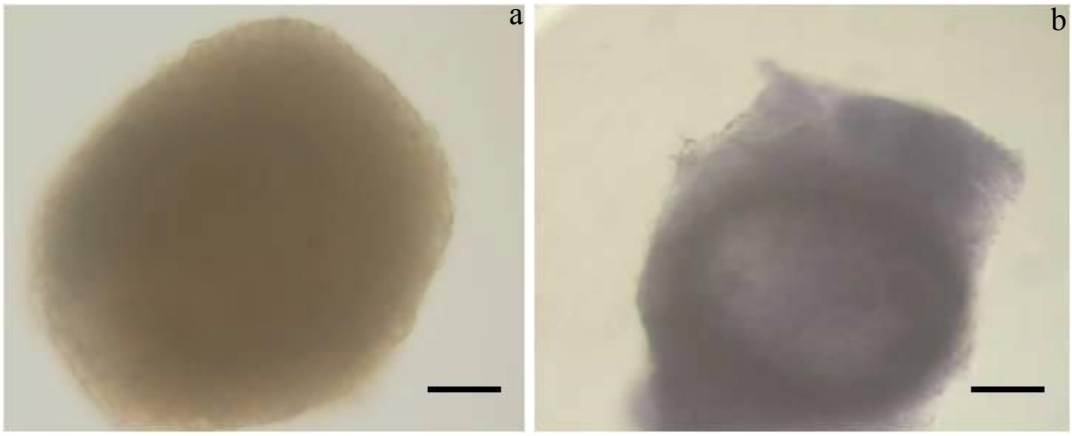

Figure 2. Photomicrographs of live and dead preantral follicles: (a) follicle showing dark, blue color, considered as dead: (b) follicle not stained, considered as live (original magnification of photomicrographs was 200X).

Influence of FSH on estradiol secretion by small and large sizes buffalo PFs

Irrespective of the FSH concentration, follicles cultured in medium containing FSH produced higher concentration of estradiol compared to the control. Small size PFs cultured with 1.0 µg/ml FSH produced more (P < 0.05) estradiol throughout the culture compared to the 0.5 and 2.0 µg/ml FSH groups. FSH 2.0 µg/ml treatment caused an inhibitory (P < 0.05) effect on estradiol secretion throughout the culture period (Fig. 3). Large sized PFs showed no significant difference in estradiol concentrations between the 0.5 and 1.0 µg/ml FSH groups throughout the culture period (Fig. 4). The supplementation of FSH (1.0 µg/ml) in the culture medium produced more (P < 0.05) estradiol compared to the control and 2.0 µg/ml FSH groups. Estradiol levels in the control medium on days 6 and 8 in small and large sizes PFs were below the sensitivity limit of the assay (4.5 pg/ml). This observation further supported that an optimal dose of FSH enhances the secretion of estradiol.

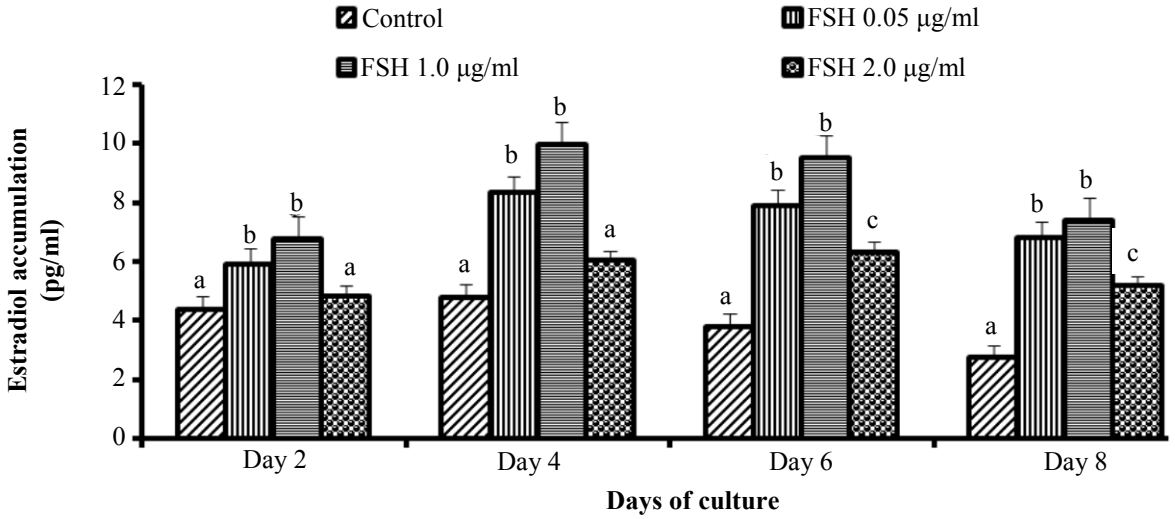

Figure 3. Influence of different concentrations of FSH on estradiol secretion by small buffalo preantral follicles cultured in vitro. Within each day, bars with different superscripts were significantly different (P < 0.05).

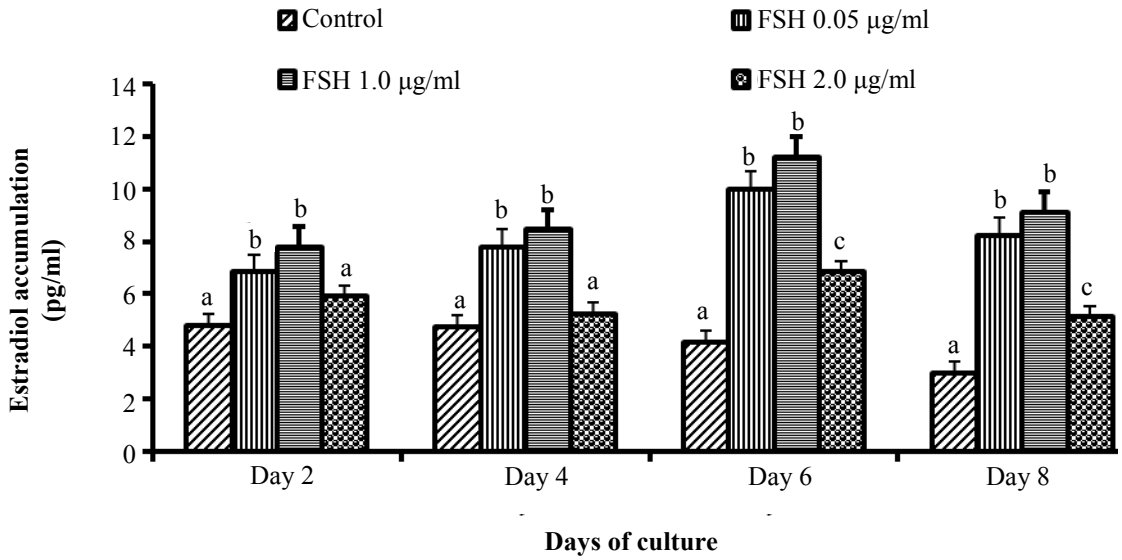

Figure 4. Influence of different concentrations of FSH on estradiol secretion by large buffalo preantral follicles cultured in vitro. Within each day, bars with different superscripts were significantly different ($P < 0.05$).

Influence of FSH on progesterone secretion by small and large sizes buffalo PFs

In vitro secretion of progesterone in the culture medium was greatly influenced by the presence of FSH compared to the control. PFs treated with 0.5 and 1.0 µg/ml FSH produced higher ($P < 0.05$) concentrations of progesterone compared to the control and 2.0 µg/ml FSH groups on days 4, 6 and 8 of culture for small PFs (Fig. 5). In large size PFs, progesterone concentrations were significantly different among different treatment groups, except on day 2 of culture. On day 8 of culture, 1.0 µg/ml FSH produced more ($P < 0.05$) progesterone compared to other groups. However, on days 4 and 6 of culture, 0.5 and 1.0 µg/ml FSH induced a higher ($P < 0.05$) secretion of progesterone compared to 2.0 µg/ml FSH (Fig. 6).

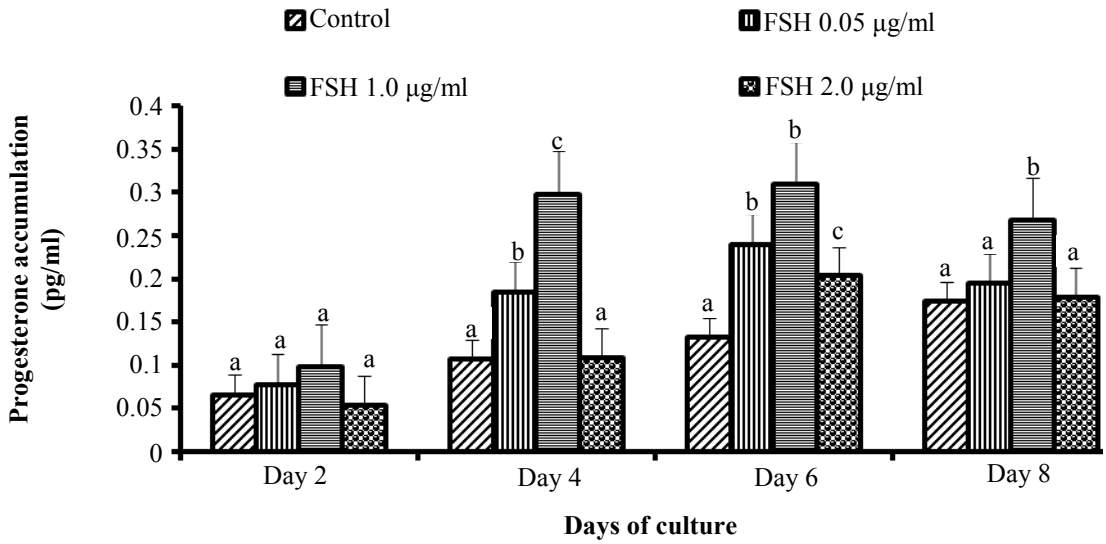

Figure 5. Influence of different concentrations of FSH on progesterone secretion by small buffalo preantral follicles cultured in vitro. Within each day, bars with different superscripts were significantly different ($P < 0.05$).

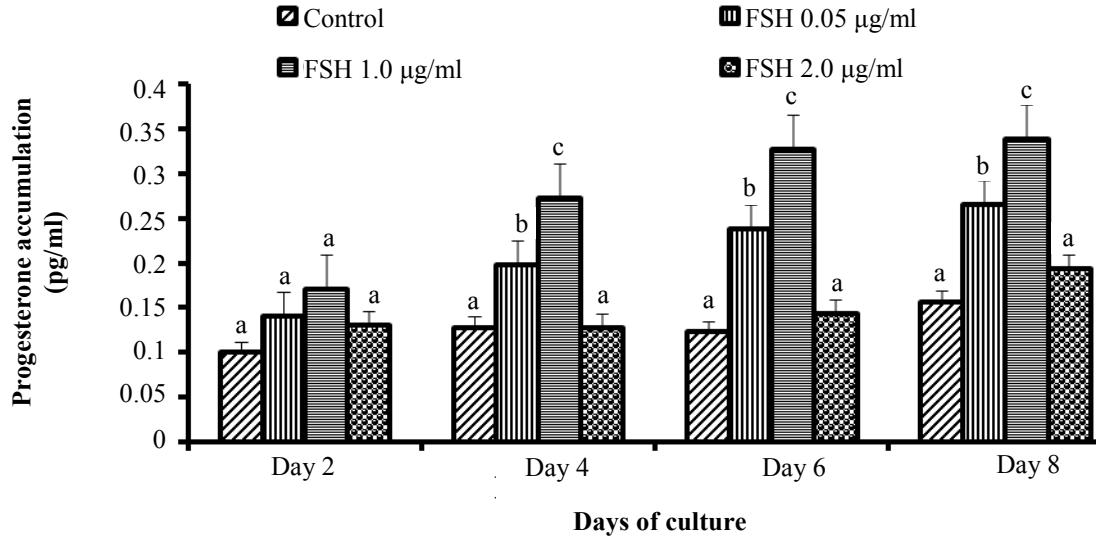

Figure 6. Influence of different concentrations of FSH on progesterone secretion by large buffalo preantral follicles cultured in vitro. Within each day, bars with different superscripts were significantly different ($P < 0.05$).

Influence of FSH on total DNA content of small (<150 µm) and large (150-300 µm) sizes buffalo PFs

The DNA content of PFs in all the treated and control groups increased with growth. The addition of 1.0 µg/ml FSH in the culture medium significantly ($P < 0.05$) increased the total DNA content (912 ± 144 ng) in small PFs compared to other treatment groups on day 10 of culture. However, the total DNA content of an individual PF cultured in the control group (380 ± 22 ng) did not differ significantly from the PFs cultured with 2.0 µg/ml FSH (418 ± 42 ng; Table 3). In the case of large size PFs, 0.5 and 1.0 µg/ml FSH produced significantly ($P < 0.05$) higher amounts of DNA content (2884 ± 615 and 6139 ± 1166 ng) compared to the control and 2.0 µg/ml FSH (1290 ± 170 and 1407 ± 117 ng) groups on day 10 of culture (Table 4).

Table 3. Influence of different concentrations of FSH on total DNA content of small buffalo preantral follicles.

Treatment	Total DNA on day 0 (ng) Mean ± SEM	Total DNA on day 10 (ng) Mean ± SEM
Control	327 ± 35[a]	380 ± 22[a]
FSH 0.5 µg/ml	327 ± 35[a]	663 ± 69[b]
FSH 1.0 µg/ml	327 ± 35[a]	912 ± 144[c]
FSH 2.0 µg/ml	327 ± 35[a]	418 ± 42[a]

[a,b,c]Within columns, means with different superscripts were significantly different ($P < 0.05$).

Table 4. Influence of different concentrations of FSH on total DNA content of large buffalo preantral follicles.

Treatment	Total DNA on Day 0 (ng) Mean ± SEM	Total DNA on Day 10 (ng) Mean ± SEM
Control	1134 ± 109[a]	1290 ± 170[a]
FSH 0.5 µg/ml	1134 ± 109[a]	2884 ± 615[b]
FSH 1.0 µg/ml	1134 ± 109[a]	6139 ± 1166[c]
FSH 2.0 µg/ml	1134 ± 109[a]	1407 ± 117[a]

[a,b,c]Within columns, means with different superscripts were significantly different ($P < 0.05$).

Discussion

The widespread implementation of advanced reproductive technologies could provide greater access to the female germ plasm. The lack of supply of fertilizable oocytes for *in vitro* manipulation is a major limiting factor of these techniques in farm animals (Van den Hurk *et al*., 2000; Kane *et al*., 2003; Van den Hurk and Santos, 2009). The isolation, culture and growth of the most abundant follicular stages in the ovary, namely

the primordial and preantral follicles, up to the Graafian follicle stage and subsequent ovulation *in vitro* can serve as an additional source of fertilizable oocytes to produce embryos. Follicle stimulating hormone is known to have divergent effects on follicular growth and differentiation in mammals both *in vivo* and *in vitro* (Zeleznik, 1993). Most of the studies have shown that FSH is involved in the growth and development of cattle (Gutierrez *et al.*, 2000; Itoh *et al.*, 2002), goat (Matos *et al*., 2007; Saraiva *et al*., 2011) and buffalo (Taru Sharma *et al.*, 2011) preantral follicles. While many studies dealing with *in vitro* systems for follicle growth indicate the growth promoting effects of FSH, it is still unclear whether or not exposure to different gonadotropins influences steroidogenesis, growth and oocyte quality for further embryonic development.

In the present study, the sensitivity of the early preantral follicle stages to FSH was clearly observed throughout the culture period. The supplementation of FSH (1.0 µg/ml) in the culture medium supported follicle survival, growth, antrum formation and enhanced DNA synthesis and estradiol and progesterone secretion in both sizes of buffalo preantral follicles. Our results suggest a dose dependent action for FSH (0.5 and 1.0 µg/ml *vs*. 2.0 µg/ml) on granulosa cell proliferation in preantral follicles. The difference in the dose of FSH in the present study and in other studies which support the best development of PFs may also be due to the difference in the source of FSH and/or culture medium. Furthermore, when FSH was omitted from the culture (control), follicle growth, survival and steroidogenesis were extremely low and normal development was retarded. These results clearly suggest that FSH promotes granulosa cell proliferation in the early preantral phase and also show that an optimal essential dose of FSH is required to allow for normal folliculogenesis. Saraiva *et al*. (2011) reported that increased concentrations of FSH have a significant impact on the *in vitro* development of caprine preantral follicles. Cecconi *et al.* (1999) reported that antral cavities were formed in sheep preantral follicles with high secreted levels of E2 in a serum-containing medium with 1.0 µg/ml FSH in a low-oxygen environment (5% O2). The data presented here confirm that the presence of FSH during the preantral phase could be important to ensure a critical number of granulosa cells to allow final survival and growth. The addition of LH in the presence of FSH might have had a beneficial effect on growth by enhancing antrum formation and supporting the development of early antral follicles. However, the role of LH during the development of preantral follicles has not yet been reported. Therefore, in the present study, LH was not included because several studies have indicated that LH is involved mainly in the development of advanced staged antral follicles. Shanmugam *et al.* (2010) demonstrated a serum-free culture system for buffalo granulosa cells and reported a stimulatory effect of FSH, but not LH, on steroid hormone production by buffalo granulosa cells. Roy and Greenwald (1988) also reported that LH suppressed FSH-induced DNA synthesis in hamster preantral follicles. Further research is required to understand the mechanism by which LH influences FSH and whether or not it stimulates aromatase activity and DNA synthesis in buffalo preantral follicular granulosa cells. The total DNA content in the cultured PFs significantly increased compared to freshly isolated PFs. The incremental diameter of the follicles indeed corresponded to an increase in cell number and hence increased the DNA content. Similar results were also reported in mice (Heise *et al.*, 2005), rats (Rowghani *et al*., 2004) and goats (Rossetto *et al.*, 2009). These results suggest that FSH acts directly as a primary stimulus at the level of small primary and secondary follicles to regulate DNA synthesis and perhaps the growth and differentiation of granulosa cells. In the present study, FSH-induced follicular DNA synthesis in a dose-dependent manner suggests that FSH induces granulosa cell proliferation, with cAMP as one of the possible intracellular mediators of FSH action in initiating DNA replication.

Estrogen and progesterone are well known endocrine and intrafollicular autocrine mitogenic compounds (Tonetta and Dizerega, 1989). Lima-Verde *et al*. (2010) reported that the interaction between estradiol and FSH maintains ultrastructural integrity and stimulates activation and further growth of cultured caprine preantral follicles. In our study, FSH (1.0 µg/ml) induced estradiol and progesterone production in all the culture groups of both follicle sizes throughout the culture period, whereas a higher amount of FSH (2.0 µg/ml) inhibited this action. Regardless of the FSH concentration, follicles cultured in medium containing FSH produced higher concentrations of progesterone and estradiol in comparison with those cultured without FSH. FSH also induced a significantly higher expression of mRNA for P450 aromatase enzymes (Silva and Christopher, 2000). FSH infusion in the physiological range appears to be the stimulus to initiate the expression of mRNA for P450 aromatase in granulosa cells. It is also possible that FSH modulates aromatase activity at two levels: transcription and translation/enzyme activity. It was observed that estradiol and progesterone secretion by small size preantral follicular granulosa cells are less sensitive than in large preantral follicles with regard to the stimulatory effects of FSH. The mechanism(s) by which buffalo preantral follicular granulosa cells from large follicles are able to respond in a more sensitive way to FSH than small follicles and also the down regulation of steroid secretion after the active period requires further study.

It can be concluded that FSH plays a key role during the development of buffalo ovarian preantral follicles as it stimulated *in vitro* survival, growth rate, steroidogenesis and DNA synthesis at an optimal dose of 1.0 µg/ml FSH.

Acknowledgments

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