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Influence of gonadotropins on steroidogenesis by large follicle buffalo granulosa cells under *in vitro* serum-free conditions

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ABSTRACT

The study was made to examine the effects of FSH and LH on estradiol–17β and progesterone production by large follicle derived buffalo granulosa cells cultured under completely serum-free conditions. Granulosa cells (3×10^5) from large (≥9 mm diameter) follicles were cultured for 24 h in 48-well plates coated with 3.3μg/cm^2 fibronectin in DMEM: Nutrient mixture F-12 Ham (1:1 ratio) supplemented with 10^{-7} M androstenedione, 5 mg/ml human apo-transferrin and 0.1% BSA, in the presence or absence of FSH or LH (1 to 64 ng/ml each). FSH had no effect on estradiol-17β output but at 4 and 8 ng/ml increased progesterone secretion by granulosa cells. All doses of LH tested had stimulatory effect on estradiol-17β while progesterone secretion by granulosa cells was unaffected.

Key words: Buffalo, Serum free culture, Steroidogenesis, Granulosa cell

Relatively poor success of embryo transfer technology (ETT) when applied to buffalo compared to their success in cattle and a number of studies in buffalo, with a focus on reproductive endocrinology, clearly brought out the differences between buffalo and cattle. Hormones and growth factors, both arising within the ovary and elsewhere profoundly affect the development of ovarian follicles, which bear the female germ cell and the surrounding granulosa and theca cells. At the ovarian level, the granulosa cells are perhaps the most important among all types of cells. These cells contribute to follicle formation and provide proper microenvironment and cytoarchitectural support for the developing oocyte. The production of steroid hormones by the granulosa cells is primarily under the control of the pituitary hormones FSH and LH. Whereas FSH exerts a major control over steroidogenesis, besides stimulating an increase in the number LH receptors during the early stages of folliculogenesis, the control of steroidogenesis shifts towards LH during the later stages of follicular development. In buffalo, reports are available on progesterone production by granulosa cells cultured either with fetal bovine serum (FBS; Bhushan *et al.* 2005) or on FBS-pre-coated plates (Bhushan *et al*. 2004). Shanmugam *et al*. (2010) reported the development of a completely serum-free culture of buffalo granulosa cell and the effect of gonadotropins on the steriodogenesis by small follicle derived granulosa cells. The present study was undertaken to examine the effects of FSH and LH on steroid production by buffalo granulosa cells derived from large follicles and cultured under completely serum-free conditions.

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MATERIALS AND METHODS

Isolation of granulosa cells: Buffalo ovaries were collected immediately after slaughter in an abattoir and washed 3 times with chilled (4°–10°C) isotonic saline (0.9% NaCl) containing 100 I.U/ml penicillin and 100 μg/ml streptomycin. The ovaries were washed 3 times with saline at room temperature after transporting to the laboratory within 5–6 h of slaughter in chilled saline. Henceforth, the ovaries were kept at room temperature until the collection of granulosa cells was completed. Granulosa cells were aspirated from large (≥9 mm diameter) follicles with a 24 gauge needle attached to a 10 ml syringe, and were washed 3 times with the culture medium which consisted of DMEM:nutrient mixture F-12 Ham (1:1 ratio) supplemented with L-glutamine (at a concentration recommended by the manufacturer), 10^{-7} M androstenedione, 5 mg/ml human apo-transferrin, 0.1% bovine serum albumin (BSA, w/v), 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.25 mg/ml amphotericin B. At each wash, the cells were separated by centrifugation at 500 × g for 10 min. The pellet was resuspended in approximately 4 ml of culture medium and

was then passed through a 30 gauge needle attached to a 5 ml syringe for breaking up clumps of granulosa cells. The cells thus obtained were primarily antral (luminal) granulosa cells. The proportion of viable cells in the granulosa cells collected, which was determined by trypan blue exclusion method using a haemocytometer, varied between 40 to 55% among different experiments.

Effects of FSH and LH: Granulosa cells were cultured in 48-well culture plates coated with fibronectin (3.3µg/cm^2) by incubation at 37°C for 1 h, at a cell concentration of 3×10^5 viable cells in 400 µl of culture medium at 37°C in a CO_2 incubator (5% CO_2 in air, 90–95% relative humidity) (Shanmugam *et al.* 2010). For studying the effects of LH or FSH, cells were cultured with the respective hormone at concentrations of 0, 1, 2, 4, 8, 16, 32 or 64 ng/ml. The media samples were collected after 24 h of culture and stored at –20°C until subsequent analysis for hormone concentration. Direct radioimmunoassay, as described earlier was used for the estimation of progesterone (Bhushan *et al*. 2004) and estradiol-17β (Palta *et al.* 1996). The intra- and inter–assay coefficients of variation were <14%.

Statistical analysis: All analysis was done using SYSTAT 7.0 software package after log transformation of data. Data from different experiments are presented as mean ± SEM hormone production (ng/ml). Each experiment was repeated 3 times and in each experiment, each treatment was performed with 4 replicate culture wells. Data were compared between control and each treatment by one-way ANOVA with Fisher's LSD test in the experiments on studying the effects of FSH and LH on steroid production.

RESULTS AND DISCUSSION

Estradiol-17β secretion by granulosa cells from large follicles cultured in the presence of graded doses of FSH was not affected (Fig. 1a). In progesterone, FSH dose levels of 4 and 8 ng/ml significantly increased (P<0.01) the secretion after 24 h of culture (Fig. 1b). Estradiol-17β secretion was significantly increased (P<0.05) by all the doses of LH after 24 h of culture (Fig. 2a). However, progesterone secretion was not affected by any of the doses of LH tested (Fig. 2b).

The effect of gonodotropic hormones on large follicle granulosa cell under serum free culture conditions is reported in this paper. Similar to the finding of no effect of FSH on estradiol-17β in this study Rouillier *et al.* (1996) also could not observe any effect of FSH on estradiol-17β secretion effect on day 1 of culture but observed a quadratic response, with FSH increasing at a dose of 2 ng/ml and decreasing it at 10 ng/ml on days 2, 3 and 4 of culture. Similar biphasic effects of FSH were also reported by Berndtson *et al.* (1995). In another later study, these authors found cattle granulosa cells to be responsive to FSH in terms of increased production of estradiol-17β on all the days of a 3-day culture (Rouillier *et al*. 1998). Similarly, Yang and Rajamahendran (1998)

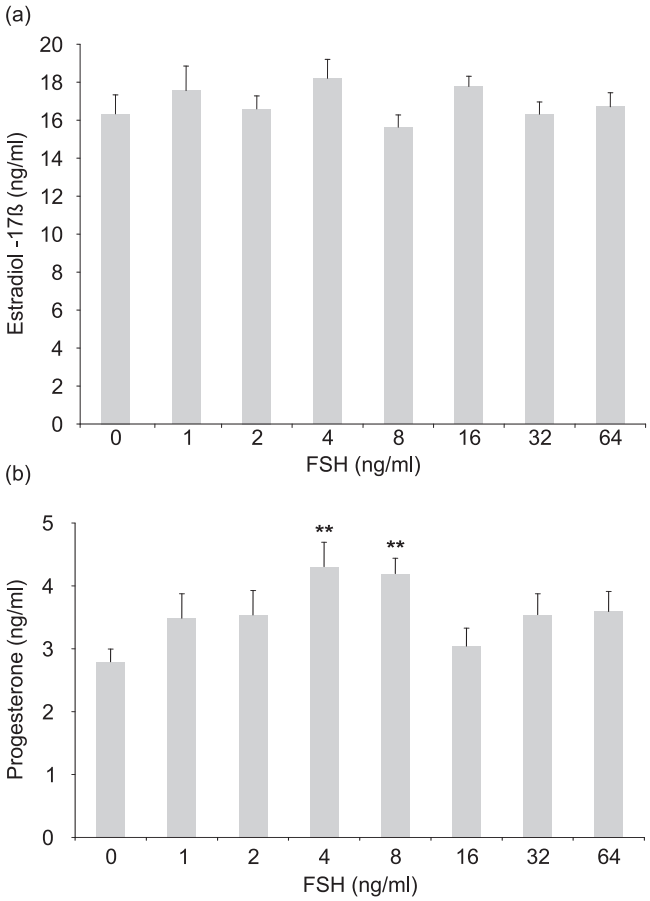

Fig. 1. Effect of FSH on (a) estradiol-17β and (b) progesterone secretion after 24 h of culture of granulosa cells under serum-free conditions. Values are mean ± SEM. Values marked with asterisks are significantly different from controls (** P < 0.01).

reported that granulosa cells from medium and large follicles were able to exhibit increased secretion of estradiol in response to FSH (1 ng/ml), but there was an inhibitory effect of FSH (10 and 100 ng/ml) on estradiol-17β output on day 2 of culture. FSH doses higher than 1 ng/ml decreased the estradiol production by granulosa cells from medium and large cattle follicles (Gutierrez *et al.* 1997). Basini and Tamanini (2000) demonstrated stimulatory effects of a high dose of FSH (100 ng/ml) on estradiol synthesis at 96 and 144 h of culture. The behaviour of the antral buffalo granulosa cells from large follicles, as observed in the present study, was similar to that of mural cattle granulosa cells in terms of not being responsive to FSH (Rouillier *et al.* 1996). Thus the effect of FSH on estradiol is not consistent and seems to vary depending on the culture conditions.

Berndtson *et al.* (1995) reported the ability of FSH to stimulate progesterone production by cattle granulosa cells from preovulatory follicles at dose levels of ≥32 ng/ml on day 1 of culture. Rouillier *et al.* (1996) also observed a linear FSH-stimulated progesterone production on day 4 of culture following treatment of cells from large follicles with 2 or 10

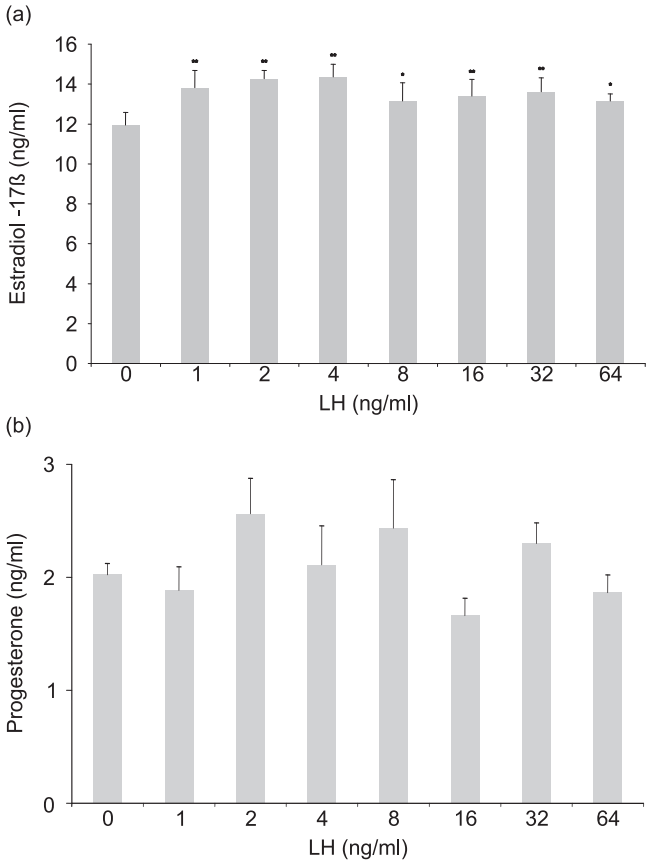

Fig. 2. Effect of LH on (a) estradiol-17β and (b) progesterone secretion after 24 h of culture of granulosa cells under serum-free conditions. Values are mean ± SEM. Values marked with asterisks are significantly different from controls (* $P < 0.05$; ** $P < 0.01$).

ng/ml FSH. FSH-stimulated progesterone production by granulosa cells from large follicles was also reported by Gutierrez *et al.* (1997) and Rouillier *et al.* (1997, 1998). However, in all these studies, the progesterone production was studied in the presence of insulin. In a study in which FSH-stimulated progesterone production was studied in the absence of insulin, Basini and Tamanini (2000) reported stimulatory effects of FSH at a high dose (100 ng/ml) on progesterone production at 48, 96 and 144 h in a 6-day culture. The present study extends the results of these studies by demonstrating stimulatory effects of FSH on progesterone secretion by granulosa cells from buffalo large follicles at physiological doses, even in the absence of insulin.

Berndtson *et al.* (1995) observed no effect of lower doses and an inhibitory effect of higher doses of LH on day 1 of culture when cattle granulosa cells from preovulatory follicles were cultured under serum-free conditions. Similarly, Yang and Rajamahendran (1998) also observed an inhibitory effect of LH (100 ng/ml) on estradiol production by bovine granulosa cells from medium and large follicles on day 1 of culture. Our results are, however, similar to those of Rouiller *et al.* (1996) who reported that LH, at doses of 12.5, 25, 50, 100 ng/ml, produced a dose dependent increase in estradiol production but only on day 4 of culture by bovine granulosa cells from large follicles. In the present study, the increased production of estradiol-17β in response to LH treatment was not dose dependent but all doses ≥ 1 ng/ml were equally effective. Under the *in vivo* conditions, granulosa cells from follicles >8 mm diameter expresses mRNA and acquires LH receptors (Xu *et al.* 1995), which then respond to the rising LH level by increasing the estradiol output.

Our result is in contrast to that of Berndtson *et al.* (1995) who found that LH stimulated progesterone secretion by bovine granulosa cells from preovulatory follicles at doses of 16-128 ng/ml on day 1 of culture. However, our results are in agreement with those of Yang and Rajamahendran (1998) who also did not find any effect of low doses of LH (1 and 10 ng/ml) on progesterone secretion by bovine granulosa cells from large follicles on days 1 and 2 of a 2-day culture.

In conclusion, large follicle buffalo granulosa cells were cultured under serum-free conditions and effect of gonadotripins on steroid secretion was tested. After 24 h of culture FSH had stimulatory effect only on progesterone secretion and LH of doses ≥ 1 ng/ml stimulated secretion of estradiol-17β but had no effect on progesterone output by granulosa cells.

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