

Effect of Follicular Fluid Proteins and Gonadotropins on Progesterone Secretion by Buffalo Granulosa Cells *In vitro*

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ABSTRACT : In the mammalian ovary the follicular fluid contains proteins and peptides which play an important role in growth, development and maturation of oocytes. The gonadotropins and some other factors work synergistically and regulate ovarian functions. In the present study the effect of follicular fluid proteins (FFP) and gonadotropins on progesterone secretion by granulosa cells (GC) from buffalo ovary, was investigated during culture. The follicular fluid was collected from small (<5 mm), and medium (5-8 mm) follicles obtained from buffalo ovaries. The follicular fluid from medium follicles was fractionated with ammonium sulphate at 80% saturation. The precipitated protein fraction was further resolved in to minor (peaks I, III) and major (peak II) proteins using gel filtration (Sephadex G-200). The FFP from small follicles and major FFP (peak II) at a dose of 200 µg/well, significantly stimulated progesterone secretion by pooled GC (3×10^5 cells/2 ml medium/well). The minor FFP did not show any stimulatory effect. There was a significant increase in progesterone secretion by pooled GC in presence of FFP and LH (10 ng/well), however, FSH (20 ng/well) with FFP exhibited an inhibitory effect. The major FFP and gonadotropins were also studied for their effect on progesterone production by GC isolated from medium and large size follicles. The GC from medium follicles were more responsive to FSH and FFP whereas GC from large follicles exhibited enhanced progesterone secretion with LH and FFP. These results indicated that FFP have their own stimulatory effect and also act synergistically with gonadotropins. The significantly different response shown by GC, for steroid hormone secretion, is based on their stage of growth and differentiation. The purification and characterization of such steroidogenic proteins may help in elucidating their role in growth and differentiation of granulosa cells. (*Asian-Aust. J. Anim. Sci.* 2004, Vol 17, No. 11 : 1496-1500)

Key Words : Buffalo Ovary, Follicular Fluid Proteins, Granulosa Cells, Progesterone, Secretion

INTRODUCTION

In mammals the ovarian follicle destined to ovulate is regulated by gonadotropins in conjunction with steroid and non-steroidal factors. Some of these factors are secreted from blood serum into follicular fluid (FF) and some are synthesized by follicular cells (Shalgi et al., 1973). They modulate the ovarian function either by stimulation or inhibition of certain biochemical processes (Kigawa et al., 1986; Shiraishi, 1986; Bendel et al., 1988). The factors with such activities were identified in ovarian extracts and follicular fluids (Sluss et al., 1988). A biologically active sheep ovarian FF peptide suppressed ovulation and decreased plasma progesterone level in normal cycling mice (Nandedkar et al., 1988a). The catecholamines identified (Trafiri, 1988) in porcine FF (pFF) and human FF (hFF) were suggested to mediate granulosa cells (GC) progesterone through gonadotropins.

The presence of a protein in hFF was reported (Khan et al., 1988) to have stimulatory effects on basal *in vitro* testosterone production by Leydig cells in rats. This protein also induced steroid production by ovarian, testicular and adrenal cells (Khan et al., 1990). The hFF contained tumor

necrosis factor, which stimulated proliferation of GC (Wang et al., 1992). Most of the studies on FF are restricted to isolation of a few proteins and peptides in human and small species. The variations in biochemical composition of ovarian FF after treatment of pigs with PMSG and effect of FF on *in vitro* development of oocytes has been reported by Huang et al. (2002). The composition was also found to vary after caprine superovulation with eCG and hCG (Mishra et al., 2003). The biochemical and hormonal characterization of ovarian follicles in small ruminants has been done in this laboratory (Singh et al., 1999). However information on role of FF in ovarian steroidogenesis in large farm animals is scanty. The present investigation describes the effect of buffalo ovarian follicular fluid proteins (FFP) on GC steroidogenesis, during culture.

MATERIALS AND METHODS

The acrylamide, bis-acrylamide, N, N, N', N'-tetramethylethylenediamine (TEMED), coomassie blue, Sephadex G-200, Medium 199 and fetal calf serum and proteins used for the preparation of standard curve were obtained from Sigma Chem. Co. (USA). The tracer 1, 2, 6, 7 [³H] progesterone was procured from Amersham, USA. FSH and LH were gift from NIH, USA. The antisera were gifted by DCP Division, NDRI, Karnal. All other chemicals used were of analytical grade.

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Collection of ovaries

The buffalo ovaries were collected from slaughter houses at Saharanpur and Delhi. The ovaries were collected in ice-cold phosphate buffer saline (0.25 M, pH 7.4) containing gentamycin (1%) and transported to the laboratory.

Isolation of follicles and follicular fluid (FF)

The collected ovaries were washed in HBSS containing glucose and phenol red. The follicles from ovaries were isolated mechanically using method of Alpizer and Spicer (1993). These follicles were grouped in small (<5 mm) and medium (5-8 mm) sizes and pooled accordingly. The individual follicles in each group size were pierced with a syringe and FF was aspirated. The pooled FF was centrifuged at 10,000 rpm for 15 min. at 4°C to remove cell debris and was stored at -20°C.

Salt fractionation

The pooled FF was fractionated with ammonium sulphate at 80% saturation and centrifuged at 3,000 rpm at 4°C for 20 min. The precipitate was dissolved in Tris-HCl buffer (0.05 M, pH 7.4) and dialyzed against the same buffer until salt was removed.

Gel filtration

According to standard procedure (Andrews, 1965), the protein sample obtained after salt fractionation was loaded on to a column (2.5×85 cm) packed with Sephadex G-200 and equilibrated with Tris-HCl buffer (0.05 M, pH 7.5). The elution was done with equilibrating buffer containing 0.1 M KCl and protein content in each fraction (3 ml) was monitored by measuring absorbance at 280 nm. The standard curve for the determination of molecular weights of FFP, was prepared using the same column (Sephadex G-200) and conditions. The standard proteins, having a wide range of molecular weights, included diamine oxidase (DAO), bovine serum albumin (BSA), ovalbumin, carbonic anhydrase (CA), trypsin and ribonuclease. The elution volume of each protein was plotted against its molecular weight.

Polyacrylamide gel electrophoresis (PAGE)

The protein profile of FF and peaks obtained after gel filtration were examined by discontinuous SDS-PAGE according to modified method of Laemmli et al. (1970).

Isolation and culturing of granulosa cells (GC)

The GC were isolated from different follicles by aspiration with the help of a syringe and collected in plain Medium 199, pooled, centrifuged and washed with medium. These cells were also isolated from medium and large follicles and pooled separately based on size of follicles.

The cell viability was determined by trypan blue exclusion method. The equal volumes of trypan blue (0.4% in normal saline) and cell suspension were mixed and counting of cells was done with a hemocytometer. The cells that did not take up the stain, were viable and those taking a bluish tinge were considered dead. The viability of cells ranged from 80-85%. The cells were cultured in culture plate (12 well), containing 3×10^5 cells/well/2 ml Medium 199, supplemented with FCS (10%), penicillin (50 IU/ml) and streptomycin (50 µg/ml), according to method of Wickings (1986).

Progesterone assay

The progesterone content in culture medium was assayed by Radio immunoassay technique modified according to Kamboj and Prakash (1993) using 1, 2, 6, 7 [³H] progesterone as tracer. The inter-assay and intra-assay variation was 11.2% and 9.8%, respectively.

Effect of gonadotropins and FFP on progesterone secretion by GC

The pooled GC were cultured in Medium 199 supplemented with FCS and antibiotics. After 48 h, the spent medium was removed and GC were washed in plain medium and cultured without FCS. At this stage (day 2), appropriate concentrations of FFP, FSH and LH were added to culture medium. After another 48 h. of culture (day 4), the spent medium was harvested and assayed for progesterone content.

Statistical analysis

The data for effect of different doses of gonadotropins and FFP on progesterone secretion were analyzed statistically using 't' test according to method of Snedecor and Cochran (1967). The FFP (200 µg), FSH (20 ng) and LH (10 ng) stimulated progesterone secretion by GC significantly ($p < 0.05$).

RESULTS AND DISCUSSION

Gel filtration profile of FFP of medium and large follicles from buffalo ovary

The protein pellet obtained at 80% saturation of ammonium sulphate resolved to three peaks, namely I, II and III as indicated in Figure 1. Some of the proteins were initially eluted in the void volume of the column and were identified as peak I. This was followed by peaks II (major) and III. The proteins resolved in these peaks showed their molecular weight in the range of 80-100, 60-80 and 40-50 kDa, respectively as extrapolated from standard curve shown in Figure 1. McGaughey and Donniel (1972) compared gel filtration of FFP and serum and reported that except for a few proteins the FFP are of serum origin.

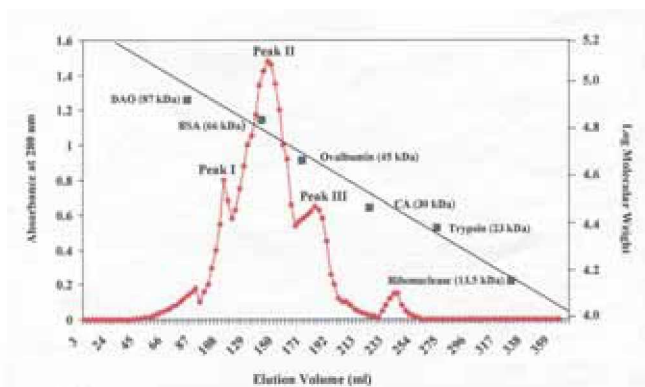


Figure 1. Gel filtration (Sephadex G-200) profile of salt fractionated follicular fluid proteins (FFP) from medium size follicles.

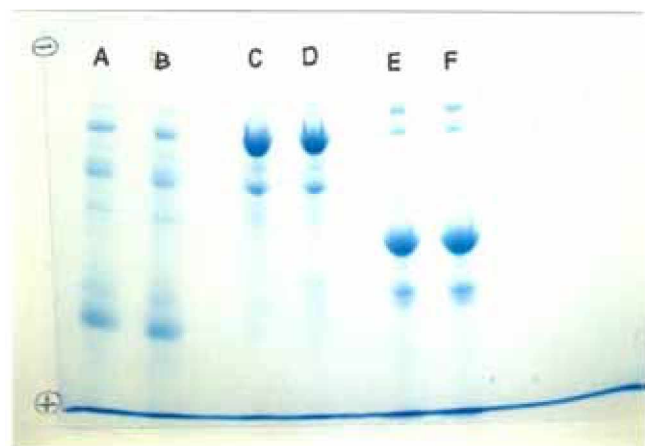


Figure 2. Comparative SDS-PAGE profile of FFP representing different peaks obtained after gel filtration. A, B: peak I, C, D: peak II, E, F: peak III.

Shalgi et al. (1973) reported that serum proteins having a molecular weight of >850 kDa can not diffuse to the FF compartment. Khan et al. (1990) obtained three protein peaks of hFF on Sephacryl S-200 column. They also got the peak I eluted in void volume, indicating the similar resolution given by two systems of matrix.

Electrophoretic profile of FFP

The fractions of peaks I, II and III obtained after gel filtration (Sephadex G-200) were pooled separately and concentrated. The proteins of these peaks showed varying number of bands on SDS-PAGE (Figure 2). These peaks contained a few major and a few minor proteins with a wide range of electrophoretic mobility. Cabrera et al. (1986) studied the electrophoretic pattern of proteins of porcine and bovine ovarian FF on SDS-PAGE and the changes in protein pattern were observed at different development stages of follicles. The FFP spectrum in small, medium and large ovarian follicles from Indian buffalo was reported (Kulkarni, 1988) indicating the consistent presence of a few

Table 1. Effect of different levels of FSH and LH on progesterone secretion by pooled granulosa cells, during culture (4 days)

Gonadotropins (ng/well)	Progesterone (pg/well)	
	FSH	LH
5	378.80±12.00	438.40±13.00
10	426.70±18.40	467.00±22.80*
20	463.60±14.40*	439.80±17.20
40	399.40±13.20	403.30±15.10
Control	335.00±12.50	

* Significant as compared to control ($p < 0.05$).

Values represented mean±SEM (n=4).

Table 2. Effect of follicular fluid proteins (FFP) from different peaks (Sephadex G-200) on progesterone secretion by pooled granulosa cells, during culture (4 days)

FFP (µg/well)	Progesterone (pg/well)		
	Peak I	Peak II	Peak III
100	350.80±14.80	417.60±13.00	349.20±21.20
200	346.60±10.00	495.20±19.20*	352.50±19.20
500	342.00±15.20	381.30±20.80	340.20±18.40
Control	348.30±13.20		

* Significant as compared to control ($p < 0.05$).

Values represented mean±SEM (n=4).

major proteins in all sizes of follicles.

Effect gonadotropins and FFP on progesterone secretion by GC

The appropriate concentrations of gonadotropins (FSH, LH) for their effect on progesterone secretion by GC were found by using a dose range of 5-40 ng/well. The maximum stimulatory effect was obtained with 20 ng of FSH and 10 ng of LH (Table 1). These doses were further used to find out their synergistic effect with FFP. The FFP peaks (I, II and III) as obtained after gel filtration were also tested for their stimulatory effect on progesterone secretion using three doses of proteins (100, 200 and 500 µg/well). As shown in Table 2, FFP from peak I and III did not exhibit any stimulatory effect on progesterone. In contrast, FFP from peak II stimulated progesterone secretion giving maximum increase (495.20±19.20 pg/well) at a dose of 200 µg protein. The peak II was a major one and represented major proteins (Figure 1) from follicular fluid. Khan et al. (1990) reported that the major protein peak (Sephacryl S-200) of hFF induced progesterone secretion by hGC. The higher concentration of peak II proteins in the present investigation was found to have little inhibitory effect due to over saturation.

In order to study the synergistic effect, the doses of 200 µg of FFP from small follicles and FFP representing peak II, were used in combination with FSH (20 ng) and LH (10 ng). There was increased progesterone production (446.40±12.00 and 526.40±16.00 ng/well) when LH was used with FFP from small follicles and peak II (Table 3), respectively. The FSH with FFP (peak II) showed some fall in progesterone production, however the value (422.00±8.60)

Table 3. Effect of FFP and gonadotropins on secretion of progesterone by pooled granulosa cells, during culture (4 days)

FFP and gonadotropins	Progesterone (pg/well)	
	Small follicles	Medium follicles
FFP (200 µg)	372.40±14.00	495.20±19.20*
FFP+FSH (20 ng)	370.60±20.80	422.00±18.60
FFP+LH (10 ng)	446.40±12.00*	526.40±16.00*
Control	320.00±23.20	348.00±13.20

* Significant as compared to control ($p < 0.05$).

Values represented mean±SEM (n=4).

remained higher than that obtained for control (348.00±13.20). Similar results were also observed by Khan et al. (1990). It may be possible that FFP from peak II contained some inhibitory factor, modulating FSH action. The inhibition of progesterone production by hFF, during GC culture and inhibition of adenylate cyclase by pFF have also been reported (Hillensjö et al., 1983). The follicular regulating protein (15 kDa) secreted by human and porcine GC inhibited GC aromatase activity (DiZerega et al., 1987). According to Kumar and Pant (1990), the injection of whole or charcoal treated buffalo FF suppressed compensatory ovarian hypertrophy in mice. The reports of various workers indicated that FF contains factors some of which stimulate steroid hormone production by follicular cells and some have inhibitory effects. A number of reports have shown that certain protein hormones and growth factors enhance progesterone secretion by granulosa cells *in vitro* in ovine, porcine, rat and cattle (Monniaux and Pisselet, 1992). However, little information is available regarding secretion of estradiol-17β by granulosa cells *in vitro*. This may probably due to difficulties in maintaining aromatase activity of granulosa cells in culture especially when serum containing media are used. Under such conditions the cells may undergo spontaneous luteinization and lose their ability to synthesize estrogens (May and Schomberg, 1981; McArdle et al., 1991). In addition, the ovarian follicular fluid has also been reported to show *in vitro* effects on the development of pig oocytes (Huang et al., 2002).

Effect of FFP and gonadotropins on progesterone secretion by GC from different sizes of follicles

The GC were isolated from medium and large follicles and cultured separately to find out the effect of FFP and FFP with FSH or LH on progesterone secretion by these GC, which represented different stages of growth and differentiation. This is evident from the results shown (Table 4) that FSH with FFP (peak II) stimulated significantly higher progesterone production (525.00±16.40 pg/well) by GC from medium size follicles over the control (387.60±14.00 ng/well). On the other hand, LH with FFP stimulated higher progesterone (537.46±15.70 ng/well) by GC from large follicles over the control. These results

Table 4. Effect of FFP and gonadotropins on secretion of progesterone by granulosa cells isolated from medium and large size follicles, during culture (4 days)

FFP and gonadotropins	Progesterone (pg/well)	
	GC from medium follicles	GC from large follicles
FFP Peak II (200 µg)	477.40±18.80	498.80±26.80
FFP Peak II+ FSH (20 ng)	525.00±16.40*	436.00±21.20
FFP Peak II+ LH (10 ng)	493.16±14.40	537.46±15.70*
Control	387.60±14.00	359.20±11.20

* Significant as compared to control ($p < 0.05$).

Values represented mean±SEM (n=4).

suggest that GC from medium follicles are comparatively more FSH dependent for steroid production whereas GC from large follicles are highly differentiated and more responsive to LH. These results are also in agreement with the findings of Asem et al. (1983), who found that GC isolated from mature follicles of turkeys produced the greatest amount of progesterone with and without LH. The present study also indicated the synergistic effect of FFP with gonadotropins. Stein et al. (1990) reported that IGF-I and insulin acted synergistically with FSH to stimulate steroidogenesis by GC from all three sizes of follicles. Similar effects were also reported by other workers including an increased progesterone secretion by pig luteal tissue with IGF-I and LH (Huang et al., 1992).

It is concluded that some major FFP have stimulatory effects on steroidogenesis by follicular cells. They also have synergistic actions with gonadotropins and exhibit modulatory functions. Though the proper mechanism of their action is not known, it may be possible that these proteins might be acting as carriers of some ligand molecules which in turn influence the process of steroidogenesis. Therefore, it is proposed that attempts should be made to purify and characterize these steroidogenic protein(s) from follicular fluid. The effect of such proteins on growth and differentiation of GC, may lead us to understand their role in ovarian functions.

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