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Effect of ovine follicular fluid peptide on follicle, oocyte, and somatic cell culture in buffalo (*Bubalus bubalis*)

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Abstract

Follicle and oocyte growth is under the control of systemic gonadotropins and various local intra-ovarian factors. The present study was undertaken to examine the efficacy of peptide(s) isolated from ovine follicular fluid on preantral and antral follicles, oocytes, cumulus and granulosa cells, and oviductal epithelial cell growth *in vitro* in a heterologous species (*Bubalus bubalis*). Follicular fluid aspirated from different follicles sizes of slaughterhouse-derived ovine ovaries was made cell free by centrifugation and steroid free by charcoal treatment. Follicular fluid was fractionated using ammonium sulphate precipitation at various saturation levels and then subjected to gel filtration chromatography. The 35-50% fraction yielded a detectable peak and a peptide of 30.1 kDa as examined by SDS-PAGE under reducing conditions. The isolated ovine follicular fluid peptide was tested for effects at different doses on *in vitro* preantral and antral follicle growth, cumulus cell expansion, *in vitro* oocyte maturation, and changes in protein, calcium, and phosphorus levels of oocytes after culture and somatic (granulosa, cumulus, and oviductal epithelial) cell monolayer formation in buffalo. The isolated peptide inhibited oocyte maturation (31% vs. 80%) and antral follicle growth (6.5 μm/day vs. 1.4 μm/day) at 1 μg/ml and 0.5 μg/ml levels, respectively, compared to control but had no effect on growth of somatic cells and preantral follicles *in vitro*. The protein, calcium, and phosphorus contents of oocytes were found to decrease in oocytes cultured in maturation medium containing the isolated peptide. The present study demonstrated the effect of an ovine intrafollicular factor regulating oocyte and antral follicle development in buffalo.

Keywords: ovine, follicular fluid, peptide, oocyte, somatic cell culture.

Introduction

Many factors in follicular fluid are involved in follicle development, oocyte growth, and follicular cell proliferation (Armstrong and Webb, 1997). The effect of these factors is difficult to examine in intact animals (*in vivo*) because of interactions between the putative direct ovarian and systemic feedback effects of such factors on gonadotropin secretion. Current knowledge of ovarian follicular peptides is restricted to IGF-binding systems, inhibin and activin, and proteoglycans; however, a large array of peptides were detected in ovine follicular fluid with qualitative but no quantitative differences in the total peptide amount related to follicle status (size, atresia, and genotype; Roche, 1996). Identification of all of these peptides and their physiological roles may lead to a better understanding of the mechanisms controlling ovarian function.

Various peptides were isolated from follicular fluid of domestic animals. A 60-kDa oocyte maturation inhibitor was isolated from bovine follicular fluid (Dostal *et al.,* 1996). A 26.6-kDa protein isolated from bubaline follicular fluid promoted the maturation of both buffalo (Gupta *et al.,* 2005b) and sheep (Gupta *et al.,* 2005a) oocytes *in vitro*. Granulosa cell inhibitory factor isolated from bovine follicular fluid suppressed folliculogenesis and granulosa cell proliferation *in vitro* in cattle (Hynes *et al.,* 1996). A 66-kDa peptide isolated from buffalo follicular fluid was found to have a stimulatory effect on steroidogenesis of granulosa cells in culture but did not have any mitogenic effect (Bansal and Sharma, 2004). Ovarian follicular fluid peptides isolated from sheep and human follicular fluid (Nandedkar *et al.,* 1988; 1996) competed with FSH in binding to granulosa cells *in vitro* and inhibited progesterone secretion from granulosa cells in culture.

Most of the earlier studies on follicular fluid were restricted to examine the effects of fractions of follicular fluid on follicle and oocyte development (Nandedkar *et al.,* 1988) and only a few on the isolation and functional characterization of the peptide from the follicular fluid (Dostal *et al.,* 1996; Gupta *et al.,* 2005a; b). Follicular fluid peptides were reported to regulate folliculogenesis (Tsafriri and Adashi, 1994) and oocyte development (Gupta *et al.,* 2005a; b) both in homologous and heterologous species. Limited research has been carried out on isolation and examination of the *in vitro* effects of isolated follicular peptides. The isolated ovine follicular peptide reported in this article was found to inhibit folliculogenesis *in vivo* in rats and inhibit follicle, oocyte, and follicular cell growth *in vitro*

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in sheep (Nandi, unpublished data). The present study was undertaken to study the effect of the ovine follicular peptide on preantral and antral follicles, the oocyte, cumulus cells, granulosa cells, and oviductal epithelial cell growth *in vitro* in buffalo (*Bubalus bubalis*), the principal dairy animal in India and a seasonal breeder like the ewe.

Materials and Methods

Material

All chemicals used for processing of follicular fluid were obtained from Sisco Research Laboratory Pvt. Ltd. (Mumbai, India) unless stated otherwise. Chemicals used for *in vitro* studies were obtained from Sigma Chemical Company (St. Louis, MO, USA). Steer serum used in the study was collected by clotting the blood of steers and inactivated with heat at 56^0 C for 30 minutes, filtered (0.22μm), sterilized, and then stored in 2 ml aliquots at -20^0C until use. The same pool of serum was used throughout the study. Ovaries used for isolation of the follicular fluid peptide were collected from healthy, cycling, non-pregnant, adult sheep (*Ovis aries*) slaughtered at a civil abattoir. Buffalo ovaries and oviducts were collected from healthy, non-pregnant, adult buffalo (*Bubalus bubalis*) slaughtered at the same abattoir.

Follicular fluid peptide isolation

Follicular fluid was aspirated from < 2, 2-4 and > 4 mm follicles of abattoir-derived sheep ovaries. The follicular fluid was made cell-free by centrifugation at 4^0C and 5000g for 30 minutes. The follicular fluid was made steroid free by charcoal treatment (McNeilly *et al.,* 1991). The supernatant follicular fluid that was used for the isolation of peptide(s) was subjected to radioimmunoassay to ascertain the presence of important gonadal steroids (testosterone, estradiol, and progesterone). The steroid hormones in the follicular fluid after charcoal treatment were found to be below detectable concentrations (<1.5 pg/ml) as estimated by radioimmunoassay. Pretreated (centrifuged and charcoal-treated) follicular fluid was fractionated with ammonium sulphate at various saturation levels: 0-20%, 20-35%, 35-50%, 50-65%, 65-75%, 75-85%, and 85-95%. The concentration of the proteins in different fractions of follicular fluid was determined by using the Bradford assay (Bradford, 1976). The maximum amount of peptide was precipitated in the 35-50% fraction. A fraction obtained at 35-50% was dialyzed (dialysis membrane; Himedia Lab. Pvt. Ltd., Mumbai, India; cut off: 12-14 kDa; pore size: 2.4 nm) against PBS for 12 hours at 4^0C under constant stirring. After dialysis, the protein sample was clarified by centrifugation (5 minutes at 5000 g) to remove debris. The supernatant was gel filtered with a Sephadex G75 (Amersham Biosciences, marketed by Sisco Research Lab. Pvt. Ltd., Mumbai, India) using PBS as the eluent. The 35-50% fraction yielded a detectable peak other than the void volume peak (peak I) after subjecting it to gel filtration chromatography. The absorbance of the fractions was read at 280 nm. Fractions constituting the eluent peak (peak II) were pooled and lyophilized. The proteins present in the eluted fractions of gel filtration chromatography were subjected to SDS-PAGE under reducing conditions (Laemmli, 1970). The gel was stained overnight with 0.25% (w/v) Coomassie brilliant blue. The molecular weight of the peptide was determined as described by Sharpiro *et al.* (1967).

In vitro assays

Effect of the isolated peptide on in vitro preantral and antral follicle growth in buffalo

The combined mechanical and enzymatic method was used to isolate preantral follicles. Thin cortical pieces were made with the scalpel blade (No. 21). The cortical pieces were then suspended in trypsin (1%; Himedia Laboratory Pvt. Ltd., Mumbai, India) prepared in normal saline, placed in 15-ml screw cap tubes, and incubated at 37^0C for 10 minutes. The cortical pieces were manipulated with needles (26 gauge) and scalpel blades under a zoom stereomicroscope, and the preantral follicles were separated from the ovarian stroma. The preantral follicles were washed in isolation and washing medium containing minimum essential medium (MEM) supplemented with bovine serum albumin (BSA, 0.3%), glutamine (2 mM), sodium pyruvate (0.23 mM), hypoxanthine (2 mM), and gentamicin (50 μg/ml). The diameter of preantral follicles was measured using a precalibrated micrometer. A follicle with an oocyte surrounded by granulosa cells and limited by an intact basement membrane was considered morphologically normal. Only large preantral follicles (200-450 μm) with normal appearances and without visible signs of degeneration were selected for culture.

The isolated preantral follicles (2 to 5 per group) were transferred in 100 μl droplets of culture media under liquid paraffin oil into a 35-mm Petri dish (Tarsons, Kolkata, India) and cultured in a CO_2 incubator (38.5 ^{o}C, 5% CO_2 in air, 90 to 95% relative humidity) for 15 days (Gupta *et al*. 2002a). The composition of control culture media was MEM supplemented with BSA (0.3%), glutamine (2 mM), sodium pyruvate (0.23 mM), hypoxanthine (2 mM), insulin-transferin-selenium (1%), FSH (0.05 units/ml), and gentamicin (50 μg/ml). The isolated peptide was added at concentrations of 0.01, 0.1, 1, or 2 μg/ml. The media were replenished every alternate day. Preantral follicle growth was measured by calculating follicular diameter using a precalibrated ocular micrometer. Morphological criteria like layers of compact granulosa cells around the oocyte, normal/abnormal follicular

outline, the presence/absence of dark patches within the granulosa membrane, and signs of degeneration (if any) for assessment of preantral follicles under the steriozoom microscope and inverted microscope were used for *in vitro* preantral follicle growth determination (Gupta *et al.,* 2002a). The preantral follicles were checked for viability using trypan blue staining technique (Gupta *et al.,* 2002a).

For isolation of the antral follicles, buffalo ovaries were sliced and the small antral follicles (1-2 mm) were dissected out in the Petri dish containing the isolation and washing medium. A follicle with an oocyte surrounded by granulosa cells and limited by an intact basement membrane was considered morphologically normal (Nandi *et al.,* 2006). The isolated antral follicles (2 to 5 per group) were transferred in 200 µl droplets of culture media under liquid paraffin oil into a 35-mm Petri dish. They were then cultured in a CO_2 incubator (38.5 ^{o}C, 5% CO_2 in air, 90 to 95% relative humidity) for 7 days. The composition of control culture media was the same as used for culture of preantral follicles. The peptide isolated was added at the following concentrations: 0.01, 0.1, 0.5, 1.0, or 2.0 µg/ml. The media was changed on Day 3 and Day 5 of culture. The evaluation of growth of antral follicles was similar to that adopted for evaluation of preantral follicles.

Effect of isolated peptide on in vitro oocyte maturation in buffalo

Oocytes were retrieved from the medium and large ovarian follicles by aspiration (Raghu *et al.,* 2002). The oocytes were checked for viability using the trypan blue staining technique (Gupta *et al.,* 2002b). Oocytes that did not take the stain were considered viable. The viable oocytes were washed thrice in TCM-199 supplemented with 0.3% BSA, and the oocytes with more than 3 layers of cumulus cells and homogenous ooplasm were only selected for culture (Chauhan *et al.,* 1998). Tissue culture medium 199 + BSA (0.3%) + FSH (0.05 units/ml) were used to create the control medium (BSA-FSH control). The peptide isolated was added to the BSA-FSH control group at the following concentrations: 0.01, 0.1, 0.2, 0.5, 1, or 2 µg/ml. Oocytes were also cultured in TCM-199 + BSA (0.3%) that served as the BSA-control. The oocytes (6-10 oocytes per group per droplet) were then transferred into 50 µl droplets of culture media in a 35-mm Petri dish. The droplets were covered with paraffin oil, and the Petri dishes were placed in a CO_2 incubator (38.5 ^{0}C, 5% CO_2 in air, 90-95% relative humidity) for 24 hours.

The evaluation of cumulus expansion of the oocytes was based on the visual assessment of the degree of expansion (cumulus expansion score) using a zoom stereomicroscope (Kobayashi *et al.,* 1994): Degree 0 - no expansion; Degree 1 - moderate expansion, cumulus cells were non-homogeneously spread, and clustered cells were still observed; and Degree 2 - fully expanded, cumulus cells were homogeneously spread and clustered cells were no longer present. Oocytes with moderate and fully expanded cumulus cell masses and unexpanded oocytes with an extruded first polar body in the perivitelline space were considered mature (Nandi *et al.,* 2002). The viability of the oocytes was determined by the trypan blue exclusion test and was in the range of 90-100%.

Estimation of protein, calcium, and phosphorus profiles in oocytes before and after in vitro culture

The oocytes were stripped of the cumulus cells by repeated pipetting after 24 hours of culture. The oocytes were then washed in 0.9% saline solution and sonicated. To compare the biochemical constituents of immature oocytes with mature oocytes, immature buffalo oocytes were also stripped of cumulus cells and sonicated. The total protein, calcium, and phosphorus profiles of oocytes before and after culture were estimated by a clinical analyzer (Photometer, Erba-Chem-5 Plus; Transasia, Mumbai, India).

Effect of the isolated peptide on granulosa, cumulus, and oviductal cell growth in buffalo

Granulosa and cumulus cells were processed according to the procedure of Wu *et al.* (2002) and Nandi *et al.* (2001), and oviductal epithelial cells were processed according to the procedure of Nandi *et al.* (2001). The viability of the cells was determined by the trypan blue exclusion test and was in the range of 85-90%. Granulosa cells (1.0 X 10^5/droplet), cumulus cells (1.0 X 10^5/droplet), and oviductal epithelial cells (~100 cells/droplet) were then washed in TCM-199 supplemented with 0.05 units/ml FSH-P and 0.3% BSA (control medium) and cultured in 50, 50, and 100 µl droplets, respectively. The peptide isolated was added to the control medium at the following concentrations: 0.01, 0.1, 0.5, 1, or 2 µg/ml. The cells were also cultured in TCM-199 supplemented with 0.05 units/ml FSH-P and 10% steer serum (serum control). The droplets were covered with paraffin oil and incubated in CO_2 incubator at 38.5 ^{0}C for 5 days for granulosa and cumulus and 7 days for oviductal epithelial cells. Media were refreshed once on Day 3 of the culture. The monolayer formation in granulosa and cumulus was evaluated for 5 days and scored according to the following criteria: Score 3 - monolayer formation started by Day 2 and covered most of the droplet by Day 5; Score 2 - monolayer formation started by Day 3 and covered more than 50% of the droplet by Day 5; Score 1 - monolayer formation started by Day 4 and covered less than 50% of the droplet by Day 5; or Score 0 - no monolayer formation by Day 4. The monolayer formation in oviductal epithelial cells was evaluated for 7 days and scored according to the following criteria:

Score 3 - monolayer formation started by Day 3 and covered most of the droplet by Day 7; Score 2 - monolayer formation started by Day 4 and covered more than 50% of the droplet by Day 7; Score 1 - monolayer formation started by Day 5 and covered less than 50% of the droplet by Day 7; or Score 0 - no monolayer formation by Day 5.

In another experiment, granulosa cells were cultured for 2 days, harvested, and cell proliferation was monitored by counting cells using a haemocytometer (Hynes *et al.,* 1996) and by an image analysis system (Image Pro-Plus; Media Cybernetics Inc., Silver Spring, USA). The viability of the cells was determined by the trypan blue exclusion test and was in the range of 75-85%.

Statistical analysis

Differences between the cumulus expansion scores, maturation rates of oocytes, preantral follicle growth, somatic cell monolayer scores, and biochemical constituents of oocytes (*in vivo* assays) were analyzed by one-way ANOVA followed by Dunnett's multiple-comparison test to compare each of the treatments with the control. The percentage values were transformed by arcsine square root before analysis. A computer assisted statistical software package (Graph Pad Prism, San Diego, USA) was used for analyzing the data. Significance of differences between mean values was determined at $P < 0.05$.

Results

Isolation of Peptide

Gel filtration chromatography of the 35-50% fraction of follicular fluid resulted in an elution peak other than the void volume peak (Fig. 1). The SDS-PAGE profile of the lyophilized peptide of the peak fraction showed a single band at the molecular weight of 30.1 ± 0.12 kDa (Fig. 2). The average yield of peptide was 0.322 mg/ml of follicular fluid.

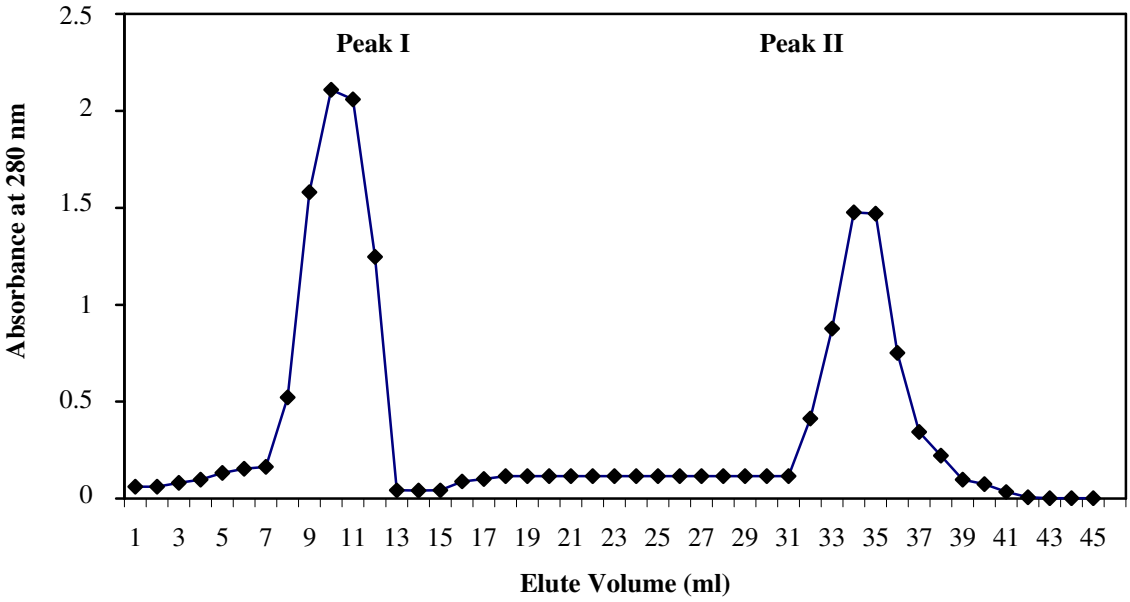

Figure 1. Elution profile of G-75 chromatographic separation of the Fraction C (35-50% saturated) of pooled follicular fluid from different follicles sizes. Peak I represents the void volume, and Peak II contains the follicular fluid peptide used in these studies.

There was no effect of treatment with different doses of follicular fluid peptide on the growth and survival of preantral follicles (Table 1). No antral cavities were observed after 15 days of culture. However, there was a significant ($P < 0.05$) inhibitory effect on the growth of antral follicles using 0.5, 1.0, and 2.0 microgram doses of follicular fluid peptide (Table 2). About 74-78% and 65-71% of cultured preantral and antral follicles survived after culture, respectively. There was no difference in survivability of preantral and antral follicles between control and treatment groups.

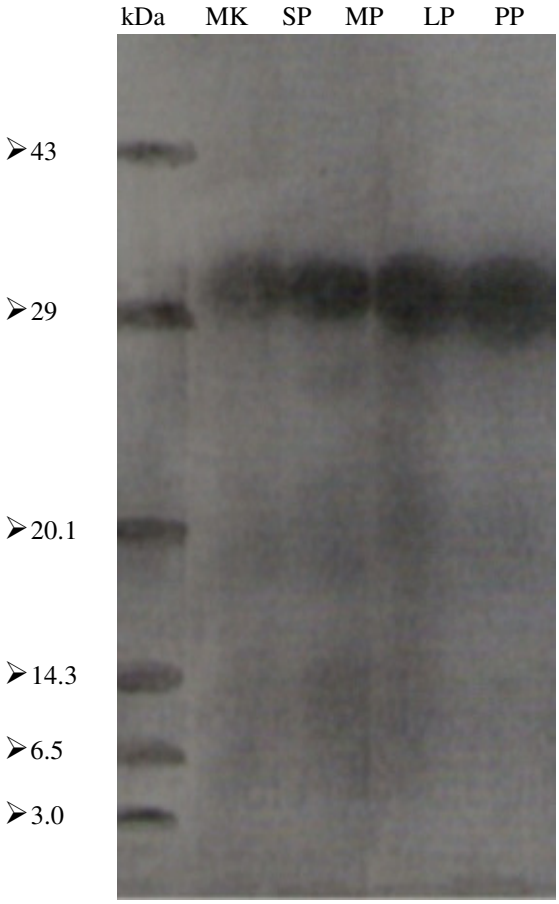

Figure 2. SDS-PAGE profile of the isolated follicular fluid peptide separated from the 35-50% fraction of follicular fluid of small, medium, and large ovarian follicles (SP, MP, and LP: isolated peptide from follicular fluids of small, medium, and large follicles, respectively; PP: isolated peptide from pooled follicular fluid; MK: standard molecular weight marker proteins; kDa: kilo Dalton).

Table 1. Effect of isolated peptide (µg/ml) on preantral follicle growth *in vitro* in buffalo.

Treatments	Follicles cultured	Follicle diameter (µm)		Growth rate (µm /day)	Survival rate (%)
		Day 0	Day 15		
Control	33	319.9 ± 12.2	348.3 ± 12.1	1.89 ± 0.4	75.7 ± 1.3
0.01 µg peptide	31	306.7 ± 9.9	333.7 ± 10.6	1.80 ± 0.3	74.1 ± 1.2
0.1 µg peptide	32	338.7 ± 11.6	363.7 ± 10.8	1.6 ± 0.2	78.1 ± 1.5
1 µg peptide	30	331.6 ± 13.2	358.0 ± 12.4	1.7 ± 0.4	76.6 ± 1.1
2 µg peptide	30	308.6 ± 11.7	332.6 ± 12.5	1.6 ± 0.3	76.6 ± 1.3

Data are expressed as the mean ± SEM. Data from 10 replicates were pooled.

Effect of the isolated peptide on cumulus expansion score and maturation of in vitro cultured buffalo oocytes

The isolated peptide inhibited oocyte maturation (31% vs. 80%) at 1 µg/ml compared to control. Cumulus expansion, and maturation rates of oocytes were significantly decreased with 1 µg/ml of isolated peptide in culture media compared to the BSA-FSH control group (Table 3). No difference was observed in cumulus expansion score and maturation rates in oocytes cultured in the BSA-FSH control medium and in media containing 0.01, 0.1, 0.2, or 0.5 µg/ml of isolated peptide. Similarly, no difference was observed in cumulus expansion score and maturation rates in oocytes cultured in BSA control medium and in media containing 1 and 2 µg/ml of the isolated peptide.

Table 2. Effect of isolated peptide (µg/ml) on antral follicle growth in vitro in buffalo.

Treatments	Follicles cultured	Follicle diameter (µm)		Growth rate (µm /day)	Survival rate (%)
		Day 0	Day 7		
Control	24	1209.1 ± 14.4	1254.7 ± 15.6	6.5 ± 2.1[a]	66.6 ± 1.0
0.01 µg peptide	22	1432.4 ± 27.2	1480.5 ± 18.6	6.9 ± 1.1[a]	65.0 ± 1.5
0.1 µg peptide	28	1381.3 ± 24.7	1419.6 ± 23.1	5.5 ± 1.4[a]	71.4 ± 1.4
0.5 µg peptide	24	1364.5 ± 20.4	1374.3 ± 17.6	1.4 ± 0.7[b]	66.6 ± 1.0
1 µg peptide	25	1314.2 ± 17.1	1324.2 ± 18.5	1.4 ± 0.3[b]	68.0 ± 1.0
2 µg peptide	24	1482.3 ± 23.6	1490.7 ± 20.4	1.2 ± 0.3[b]	66.6 ± 1.0

Data are expressed as the mean ± SEM. Data from 8 replicates were pooled.
Values with different superscripts in the same column differ ($P < 0.05$).

Effect of the isolated peptide on protein, calcium, and phosphorus levels of oocytes after culture

The protein, calcium, and phosphorus contents were significantly higher in mature oocytes compared to immature oocytes. No difference in the calcium content was observed in oocytes cultured in BSA-FSH control media and in media containing 0.01, 0.1, 0.2, or 0.5 µg/ml of isolated peptide (Table 4). The calcium content was significantly lower in oocytes cultured in 1 µg/ml isolated peptide compared to those cultured in 0.5 µg/ml. A further significant decrease was observed in oocytes cultured in 2 µg/ml of the isolated peptide compared to those cultured in 1 µg/ml. No difference in the calcium content was observed between oocytes cultured in media containing 2 µg/ml of isolated peptide and the BSA-control medium.

Table 3. Effect of isolated peptide (µg/ml) on cumulus expansion score and maturation *in vitro* in buffalo oocytes.

Treatments	Oocytes cultured (n)	Cumulus expansion score	Oocytes matured (%)
BSA-FSH control	86	1.7 ± 0.11[a]	69 (80.2 ± 2.4)[a]
0.01 µg peptide	82	1.7 ± 0.11[a]	64 (78.0 ± 2.0)[a]
0.1 µg peptide	88	1.6 ± 0.10[a]	70 (79.5 ± 1.8)[a]
0.2 µg peptide	84	1.4 ± 0.12[a]	62 (73.8 ± 2.3)[a]
0.5 µg peptide	88	1.4 ± 0.12[a]	62 (70.4 ± 2.0)[a]
1 µg peptide	87	0.7 ± 0.02[b]	27 (31.0 ± 1.3)[b]
2 µg peptide	84	0.7 ± 0.02[b]	24 (28.5 ± 1.2)[b]
BSA- control	82	0.5 ± 0.02[b]	23 (28.0 ± 1.5)[b]

Data are expressed as the mean ± SEM.
Data from 12 replicates were pooled.
Values with different superscripts in the same column differ ($P < 0.05$).

Table 4. Effect of isolated peptide concentration (µg/ml) in oocyte culture media on changes in protein, calcium, and phosphorus contents of buffalo oocytes after culture.

Treatments	Biochemical changes in oocytes (µg/oocyte)		
	Protein	Calcium	Phosphorus
Oocyte before culture	104.1 ± 1.6[a]	1.02 ± 0.01[a]	0.90 ± 0.01[a]
	OOCYTE AFTER CULTURE		
BSA-FSH control	228.5 ± 2.8[b]	3.62 ± 0.12[b]	2.35 ± 0.02[b]
0.01 µg peptide	219.2 ± 2.7[b]	3.40 ± 0.09[b]	2.32 ± 0.02[b]
0.1 µg peptide	220.5 ± 8.0[b]	3.32 ± 0.05[b]	2.23 ± 0.02[b]
0.2 µg peptide	212.9 ± 4.9[b]	3.40 ± 0.07[b]	2.19 ± 0.02[b]
0.5 µg peptide	205.6 ± 3.9[b]	3.28 ± 0.02[b]	2.20 ± 0.05[b]
1 µg peptide	156.1 ± 1.1[c]	2.45 ± 0.03[c]	1.64 ± 0.02[c]
2 µg peptide	150.8 ± 1.7[c]	2.12 ± 0.01[d]	1.58 ± 0.01[c]
BSA- control	150.3 ± 3.3[c]	2.10 ± 0.01[d]	1.55 ± 0.02[c]

Data are expressed as the mean ± SEM.
Data from 3 replicates were pooled.
Eighty oocytes were examined in each group.
Values with different superscripts in the same column differ ($P < 0.05$).

Effect of the isolated peptide on cumulus, granulosa, and oviductal cell monolayer formation, and granulosa cell proliferation in buffalo

No difference was observed in cumulus, granulosa, and oviductal cell monolayer formation in BSA-control medium and in media containing the isolated peptide at different concentrations (Table 5). The monolayer formation was significantly higher in serum-control medium compared to BSA-control medium and media containing isolated peptide at different concentrations.

The isolated peptide had no proliferative effect on granulosa and cumulus cells with all tested concentrations. The cell concentrations (x 10^5/ml) after various treatments were as follows: control, 1.24 ± 0.11; 0.01 μg/ml peptide, 1.24 ± 0.17; 0.1 μg/ml peptide, 1.22 ± 0.16; 0.5 μg/ml peptide, 1.16 ± 0.22; 1 μg/ml peptide, 1.20 ± 0.20; and 2 μg/ml peptide, 1.16 ± 0.14.

Table 5. Effect of isolated peptide concentration (μg/ml) on cumulus, granulosa, and oviductal cell monolayer formation score in buffalo.

Treatments	Somatic cell monolayer formation score		
	Cumulus cell	Granulosa cell	Oviductal cell
BSA control	2.08 ± 0.14[a]	1.75 ± 0.13[a]	1.75 ± 0.13[a]
0.1 μg peptide	1.91 ± 0.08[a]	1.75 ± 0.13[a]	1.66 ± 0.14[a]
0.1 μg peptide	1.91 ± 0.08[a]	1.75 ± 0.13[a]	1.58 ± 0.14[a]
0.5 μg peptide	1.83 ± 0.11[a]	1.75 ± 0.13[a]	1.58 ± 0.14[a]
1 μg peptide	1.83 ± 0.11[a]	1.66 ± 0.14[a]	1.66 ± 0.14[a]
2 μg peptide	1.83 ± 0.11[a]	1.66 ± 0.14[a]	1.66 ± 0.14[a]
Serum- control	2.75 ± 0.13[b]	2.25 ± 0.13[b]	2.16 ± 0.11[b]

Data are expressed as the mean ± SEM.
Data from 12 replicates was pooled.
Values with different superscripts in the same column differ ($P < 0.05$).

Discussion

The main thrust of the current research in female reproduction is to elucidate how locally-produced factors, mainly members of the transforming growth factor β superfamily and including inhibins, activins, follistatin, and bone morphogenetic proteins, contribute to the regulation of ovarian follicular development and ovulation through both systemic (endocrine) and intra-ovarian (autocrine/paracrine) signaling actions. The present study demonstrated that a follicular fluid peptide fraction effected oocyte maturation and antral follicle growth *in vitro*. The effect of the isolated peptide was not a toxic effect, as treatment with the isolated peptide did not decrease the proportion of dead cells in culture. Elucidating the role of the isolated peptide in oocyte-somatic cell interactions at the various stages of follicle development will have important implications for our understanding of factors regulating folliculogenesis, ovulation rate, and fecundity.

The isolated peptide had no effect on the growth of preantral follicles *in vitro*. A similar observation was reported with preantral follicles of sheep (Nandi, unpublished data). The growth of preantral follicles *in vitro* was reported to be gonadotropin independent; however, FSH was shown to promote preantral follicle growth *in vitro* in buffalo (Gupta *et al.,* 2002a). Hence, FSH was supplemented in the control medium. The isolated peptide did not show any significant effect on preantral follicle growth unlike the effect on oocyte maturation. The long-term culture of small and large preantral follicles with the isolated peptide as an additive could offer a new approach for investigating the precise mechanism of regulation of follicular development. There is no published literature on the effect of any local ovarian factor on antral follicle growth *in vitro*. The present study demonstrated that the isolated peptide inhibited antral follicle growth *in vitro*. Whether or not the isolated peptide in the present study acted in concert with other locally-produced factors in antral follicle development regulation needs to be investigated. The exact mechanisms through which the isolated peptide operated and the degree of redundancy needs to be elucidated.

Factors responsible for stimulation and inhibition of cumulus cell expansion and oocyte maturation *in vitro* have been isolated from follicular fluid (Dostal *et al.* 1996). Follicle-enclosed oocytes did not resume meiosis either *in vivo* or *in vitro* in the absence of appropriate signals. This suggested that the presence of inhibitory agent(s) within the antral follicle is responsible for maintaining the oocyte in the germinal vesicle (GV) stage. Arrest of oocytes at the first meiotic division (GV stage) could be determined by the presence of the maturation-promoting factor, which is composed of two subunits (p34cdc2 kinase and cyclin B; Gordon, 2002). A heat-stable polypeptide in follicular fluid was isolated that maintained meiotic arrest in cattle oocytes; this factor was apparently derived from the somatic cells of the follicle and released into the follicular fluid (Gordon, 2002). Supplementation of the oocyte maturation medium with 2.0 mg/ml of the 60-kDa peptide isolated from bovine

follicular fluid induced meiotic arrest in all treated oocytes (Dostal *et al.,* 1996). The inhibitory activity present in porcine follicular fluid had been attributed to the presence of oocyte maturation inhibitor (OMI, Mr: 2 kDa; Tsafriri *et al.,* 1982) secreted from granulosa cells. *In vitro* studies with follistatin, an activin binding protein, found that follistatin reduced the developmental competence of *in vitro* matured bovine oocytes (Silva and Knight, 1998). The effects of inhibin on oocyte maturation have remained controversial. In rat oocytes, inhibin-α inhibited nuclear maturation (Robertson *et al.,* 1989); whereas in primates (Alak *et al.,* 1996) and cattle (Stock *et al.,* 1997), inhibin-A increased oocyte maturation. A suppressive effect of inhibin-α on the developmental competence of bovine oocytes in cattle has been demonstrated (Silva *et al.,* 1999). In the present study, oocyte maturation was inhibited with 1 μg/ml of the isolated peptide in the oocyte culture medium; in sheep though, the inhibitory effect was significant using 0.5 μg/ml (Nandi, unpublished data). These concentrations were probably different due to variation between species. It might be possible that the isolated peptide inhibited binding of FSH to receptors on cumulus cells, thereby reducing the stimulatory action of FSH on cumulus-oocyte complex maturation. A direct inhibitory effect of the peptide on granulosa and cumulus cells could also be possible. The isolated peptide had an inhibitory effect on the protein, calcium, and phosphorus contents in the oocytes in a dose dependent manner after culture. The information on the biochemical changes during maturation of oocytes is limited. The protein and phosphorus contents in oocytes after *in vivo* maturation were limited to a single study in buffalo wherein protein and phosphorus levels increased in the oocytes after culture in media containing IGF-I (Nandi *et al.,* 2005).

The present study demonstrated the effect of a 30.1 KDa ovine intrafollicular factor regulating the oocyte and antral follicle development. The isolated peptide was inhibitory for both cumulus and granulosa cell proliferation *in vitro* in sheep (Nandi, unpublished data). However, the inhibitory effect was not observed in buffalo in the present study. This suggested that the isolated peptide might not have any effect on cumulus and granulosa cell proliferation *in vitro* in buffalo, which is a heterologous species. The present study also indicated that the isolated peptide did not have any effect on oviductal cell (extra-ovarian cells) proliferation in culture. Further studies are needed to describe the amino acid sequence of the isolated peptide and to ascertain whether the effect of the peptide was an endocrine or local (autocrine and paracrine) effect.

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