Isolation of an Oocyte Stimulatory Peptide from the Ovarian Follicular Fluid of Water Buffalo (*Bubalus bubalis*)*

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ABSTRACT : Ovarian follicular fluid contains both stimulatory and inhibitory agents that influence the growth and maturation of oocyte. In the present study, an attempt was made to isolate and study the biological properties of ovarian follicular fluid peptide(s) in buffaloes. Bubaline ovarian follicular was made steroid- and cell-free. A protein fraction was obtained by saturation (30-35% level) of the follicular fluid with ammonium sulfate. The protein fraction was purified with Sephadex-G 50 gel filtration chromatography and a single peak was obtained in the eluant volume, which was lyophilized. SDS-PAGE of the lyophilized fraction revealed a single band and the molecular weight of the peptide was 26.6 kDa. The peptide stimulated the cumulus cell expansion and *in vitro* maturation rate of oocytes in buffaloes in a dose dependent manner when it was incorporated at different dose levels (0, 10, 25, 50, 100 and 1,000 ng ml^{-1} of maturation medium). The basic culture medium consisted of TCM 199 with Bovine serum albumin (0.3%). The *in vitro* maturation rates were comparable to those obtained with a positive control medium (TCM 199+20 ng EGF ml^{-1}+steer serum (20%)). Further purification and biological assays may throw more light on the nature and functions of this peptide. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 11 : 1557-1563)***

Key Words : Follicular Fluid, Peptide, Oocyte, IVM, Buffalo

INTRODUCTION

Mammalian oocytes, especially of domestic animals are in great demand due to increasing importance for cloning studies. They are used as the recipient cells for transferring the karyoplast with a selected genome. Thus a high oocyte yield from the animals would fulfill the needs of transgenic and cloning works. Efforts are being made to economize the production of mature oocytes by way of replacing the expensive components of culture media with less expensive ones. Successful *in vitro* maturation (IVM) of oocytes could be the first step in the process of laboratory embryo production. To reduce the cost of this technology, conventionally used fetal bovine serum (FBS), an important and expensive component in the *in vitro* maturation medium of oocytes is being successfully replaced with steer serum or cow serum or follicular fluid. Follicular fluid contains all soluble substances implicated in follicular differentiation, growth and oocyte maturation (Richards, 1994). Follicular fluid is being successfully incorporated in IVM media of different domestic animals i.e cattle (Larocca et al., 1993), buffalo (Chauhan et al., 1997; Tajik et al., 2000), horse (Bruck et al., 2000), sheep (Guler et al., 2000), pig (Grupen et al., 1997; Zak et al., 1997) and rabbit (Zhang and Wang, 1994).

The positive effects of follicular fluid on *in vitro* oocyte maturation are due to the presence of several peptides/ growth factors, Follicle stimulating hormone (FSH), Leutinizing hormone (LH) and several nutrients, whereas the negative effects are due to the presence of certain inhibitory factors like oocyte maturation inhibitory factor (OMI) (Dostal et al., 1996). In cattle, up to 50% replacement of generally used IVM medium with follicular fluid has resulted in high maturation rate (Ocana Quero et al., 1997) but replacement at 100% level inhibited the maturation rate (Bevers et al., 1992; Ayoub and Hunter, 1993). The ovarian follicular fluid of buffaloes is more stimulatory to *in vitro* oocyte maturation in comparison to that of other species (Gupta et al., 2001a) leading to a presumption that the follicular fluid in buffaloes might contain more stimulatory peptides. Incorporation of stimulatory peptides isolated from ovarian follicular fluid rather than the whole follicular fluid in IVM medium may reduce the chances of disease transmission.

Follicular fluid proteins play a vital role in folliculogenesis and selection of dominant follicle (Rivera and Fortune, 2003; Riley et al., 2004). The ovarian follicular fluid peptides like inhibin, granulosa cell inhibitory factor (GCIF) (Hynes et al., 1996), oocyte maturation inhibitor (Dostal et al., 1996) and follicular regulatory protein (Fujimori et al., 1987) have been isolated /characterized in domestic animals like cattle, sheep and pigs but not in buffalo. Studies on the isolation of peptides from the follicular fluid of buffalo are scarce. A peptide

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consisting of 2 polypeptide subunits (Mr 21 and 36 kDa) was isolated from the ovarian follicular fluid of buffaloes by gel filtration (Talevi et al., 1994). An inhibin like peptide was isolated from the ovarian follicular fluid of buffaloes (Kumar and Pant, 1990; Srivastava and Pant, 1999). Partially purified peptide fractions from bubaline ovarian follicular fluid stimulated the progesterone secretion from buffalo granulose cells (Mukesh Vinze et al., 2004). In this study, an attempt was made to isolate and study the biological activity of peptide (s) from the ovarian follicular fluid of water buffalo with an aim to determine the effect of the isolated peptide (s) on buffalo oocytes in order to select the isolated peptide as input in IVM.

All chemicals and tissue culture media were obtained from Sigma Chemical Co. (USA) unless otherwise stated.

Collection and pre- treatment of follicular fluid

Buffalo ovaries were obtained from the local abattoir immediately after the slaughter of buffaloes (>2 years age with unknown reproductive status) and transported to the laboratory in chilled (4°C) normal saline (0.9% Sodium chloride with gentamicin: 50 μg ml^{-1}) with in 1h of the slaughter. Ovaries were washed thoroughly with chilled normal saline in the laboratory. Follicular fluid was collected from surface follicles by using sterile disposable syringe with 22-G needle. An anti proteolytic agent i.e. Phenyl methyl sulfonyl fluoride (PMSF) was added (20 μg ml^{-1}) to follicular fluid. Follicular fluid was made cell-free by centrifugation at 4°C at 1,500×g for 30 min. It was made steroid-free by charcoal treatment. Activated charcoal (5 mg ml^{-1}) was added and the fluid was stirred for 1 h and was later centrifuged for 30 min. at 1,500×g. 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 i.e. testosterone, estradiol and progesterone.

Isolation of peptide

Pretreated follicular fluid was fractionated with ammonium sulfate (molecular biology grade of SRL Chemicals, Mumbai, India) at various saturation levels. A fraction obtained at 30-35% saturation was dialyzed (dialysis membrane with cut off Mr. 12-14 kDa, Himedia Labs, Mumbai, India) for 15 h against Phosphate buffer saline (PBS; pH 7.2) by constant stirring at 4°C. The retentate was centrifuged for 30 min. at 1,500×g. The supernatant was gel filtered with Sephadex G-50 (Amersham Biosciences, repacked and distributed by Sisco Research Laboratories Pvt. Ltd., Mumbai, India) using PBS as the eluent. The length and diameter of the column were 83 cm and 1.2 cm, respectively and the flow rate was 8 ml/ min. Fractions of 1.5 ml were collected and the absorbance of the fractions was read at 280 nm. Fractions constituting eluant peak were pooled and lyophilized.

Sodium doedecyl sulfate -Poly acrylamide gel electrophoresis (SDS-PAGE)

The lyophilized fraction constituting the eluant peak was analyzed by SDS-PAGE with 12.5% acrylamide gel under reducing conditions (Laemmli, 1970). The gels were stained with coomassie brilliant blue-R. The molecular weight of the peptide present in the lyophilized fraction was estimated by standard protein marker (PMW-M; Bangalore Genei, Bangalore, India). Similar procedure of SDS-PAGE was followed to study the peptide bands of protein fraction at the different stages of purification (ammonium sulfate precipitate of follicular fluid) and whole follicular fluid (cell free) collected from different size categories of ovarian follicles.

Western blot analysis of the isolated peptide

Antiserum was raised in rabbit against the partially purified peptide that was detected by Western Blot analysis as per procedure described by Sambrook et al. (1989).

Collection of cumulus oocyte complexes (COCs)

Ovaries collected from matured, non pregnant slaughtered buffaloes were transported to the laboratory in warm (32-33°C) normal saline (0.9% NaCl) containing gentamycin (50 μg ml^{-1}) within 1 h of slaughter. The ovaries were washed in 0.9% normal saline in the laboratory. From buffalo ovarian follicles, the oocytes were aspirated from the surface follicles (3-6 mm. diameter) using a 10 ml syringe fitted with 18-G needle. Aspiration medium consisted of TCM 199 supplemented with 10% steer serum and phosphate buffered saline (PBS) mixed with 0.3% fraction V bovine serum albumin (BSA) at 1:1 ratio. Cumulus oocyte complexes (COC) were isolated under zoom stereo microscope (Nikon, Japan). The COCs with more than five layers of compact cumulus cells and with evenly granular homogenous ooplasm were selected for the *in vitro* maturation studies.

***In vitro* maturation of oocytes**

The selected oocytes were washed three times with the aspiration medium and once with the culture medium and were then transferred into 50 μl droplets (8 to 10 COCs per drop) of culture medium. The droplets were covered with warm (38.5°C) mineral oil and then placed in a CO_2 incubator (38.5°C, 5% CO_2 in air, 90-95% relative humidity) for 24 h.

The basic oocyte culture medium consisted of Medium 199, BSA (0.3%), and Gentamicin (50 μg ml^{-1}). The isolated peptide was added to the culture medium at different doses i.e. 0, 10, 25, 50, 100 and 1,000 ng ml^{-1} of maturation medium. As a positive control the basic culture

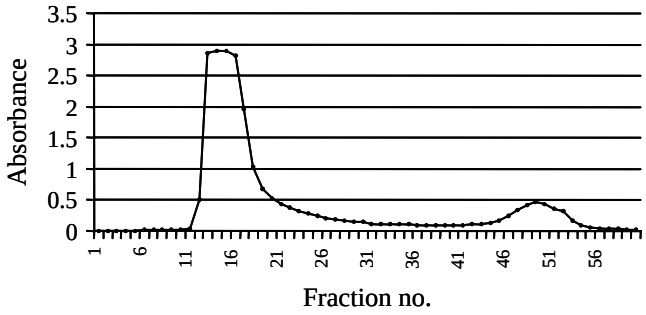

Figure 1. Elution profile of a G-50 Sephadex chromatographic separation of the ammonium sulfate precipitate (30-35% saturation) fraction of follicular fluid in buffaloes. First peak is void volume peak and the second peak is elution volume peak from which the peptide was isolated.

medium was supplemented with the steer serum (20%) and Epidermal growth factor (EGF) (20 ng ml^{-1}). After 24 h of incubation the oocytes were examined for determining the biological activity of the incorporated novel peptide in IVM medium.

Assessment of the *in vitro* biological activity of the peptide

The biological activity of the peptide at different levels of supplementation was estimated by the mean cumulus expansion score as well as by maturation rate. After 24 h of incubation the oocytes were examined for their cumulus expansion, the degree of expansion was graded for each and every oocyte based on a method adopted by Downs (1989) for rat and by Harper and Brackett (1993) for cattle. If there was no cumulus expansion at all, it was graded as 0, slight cumulus expansion involving only outer most layer as 1, partial cumulus expansion involving all layers except corona radiata as 2 and full cumulus expansion involving all layers including corona radiata as 3. The mean cumulus expansion score of oocytes in a particular group was calculated by dividing the sum of cumulus expansion grades of all the oocytes by the total number of oocytes under the treatment. Random sample of oocytes with grade 2 and grade 3 cumulus expansion were denuded and were observed for first polar body emission. First polar body was found in all the oocytes in which grade 2 or 3 cumulus expansion was noticed. Hence all the oocytes with grade 2 and grade 3 expansion were considered matured. In addition, all the grade 0 and grade 1 oocytes were also observed for the emission of first polar body. If it was found, then only they were also considered as matured for the purpose of calculating the maturation rate of oocytes (Nandi et al., 2002).

Statistical analysis

The differences among the cumulus expansion scores, *in vitro* maturation rates (the percent maturation was transformed using arcsine transformation) were analyzed by ANOVA and the respective means were compared using Bonneforni Multiple comparison test (Graph Pad PRISM, Graph Pad Software Inc., San Deigo, USA). Differences between the mean values were considered significant when the P values were less than 0.05.

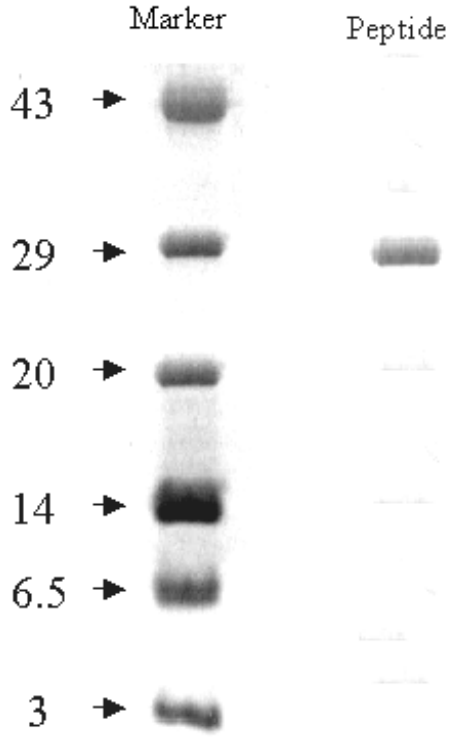

Figure 2. SDS-PAGE profile of the isolated peptide in comparison to standard marker (1st lane from left). The numbers in the Figure indicate the molecular weight (kDa) of the corresponding peptide bands of the standard protein marker.

RESULTS

Isolation of peptide

The follicular fluid that was used for the isolation of peptides was devoid of steroids after the charcoal treatment. The steroid hormones (progesterone, testosterone and estradiol) were found to be below detectable concentration (<2 pg ml^{-1} of follicular fluid) as estimated by radioimmuno assay. Nine percent of the protein present in the follicular fluid was precipitated into the fraction obtained with 30-35% level of ammonium sulfate saturation. When this protein precipitate obtained after ammonium sulfate precipitation was subjected to gel filtration, the protein recovered in the isolated peptide was only 1.5% of that was loaded in to the column. Consistently, a single protein peak was obtained in the eluant (Figure 1) when the follicular fluid peptide precipitate was gel filtered through sephadex G-50. The isolated peptide constituted the 0.13% of the protein present in the follicular fluid. The SDS-PAGE

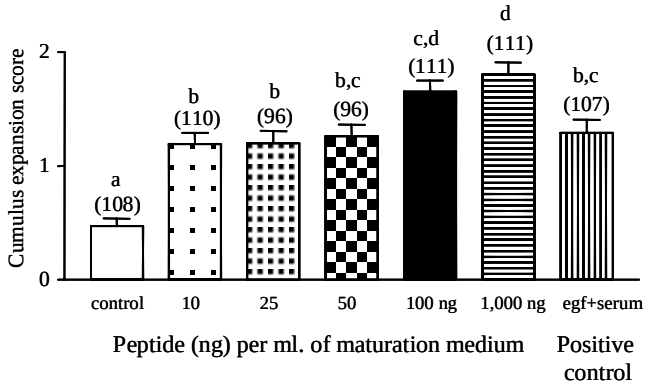

Figure 3. Effect of incorporation of the isolated peptide in the *in vitro* maturation medium on the cumulus expansion of buffalo oocytes. Values are means±SEM of cumulus expansion score of the oocytes (number in parentheses) included in a particular treatment group. Bars with different letters differ significantly ($p<0.05$). Each treatment was replicated five times.

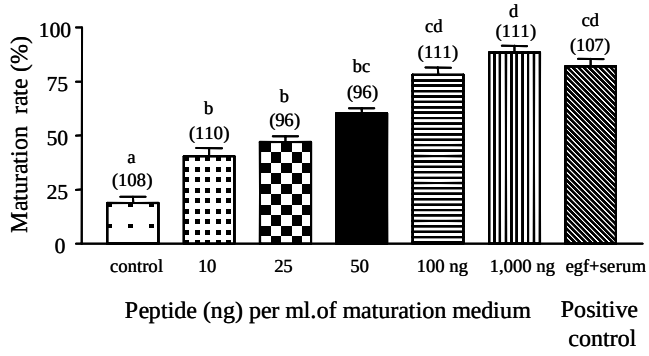

Figure 4. Effect of incorporation of the isolated peptide in the *in vitro* maturation medium on the *in vitro* maturation rate of buffalo oocytes. Values are means±SEM of *in vitro* maturation rate of the oocytes (number in parentheses) included in a particular treatment group. Bars different letters differ significantly ($p<0.05$). Each treatment was replicated five times.

profile of the lyophilized material of the peak fractions showed a single peptide band (not a sharp band) at Mr 26.6 kDa (Figure 2). Between the region of 20-29 kDa, peptide bands were observed in the SDS-PAGE profile of whole follicular fluid as well as the ammonium sulfate precipitate (30-35% saturation). The peptide of the same molecular weight was present in the fluid of all the size categories of ovarian follicles. Clear bands were visible in the western blotting with the blotted peptide from all the three categories of follicles (Figure 5).

In vitro cumulus expansion

A dose dependent increase in the cumulus cell expansion rate was observed with the isolated peptide when it was incorporated in the *in vitro* maturation medium of oocytes (Figure 3). A significantly ($p<0.05$) increased response of cumulus cell expansion rate in comparison to control group was obtained even at the low dose of 10 ng

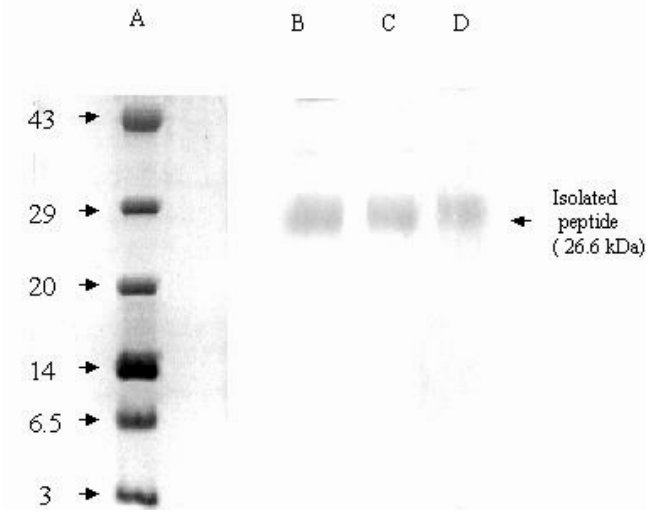

Figure 5. Western immunoblot of the isolated peptide. A: Marker, B: Peptide isolated from small follicles, C: Peptide isolated from medium sized follicles, D: Peptide isolated from large follicles.

ml^{-1}. Highest rate of cumulus cell expansion was obtained by 1,000 ng dose, which was not significantly ($p>0.05$) different from the 100 ng. dose, but was significantly more than that of positive control. There was no significant difference ($p>0.05$) in the cumulus cell expansion rate between the positive control group and the 10 ng dose group.

In vitro maturation rate

There was a dose dependent increase in the *in vitro* maturation rate (Figure 4). Like cumulus expansion score, there was a significant increase noticed in the case of *in vitro* maturation rate with the lowest dose of 10 ng ml^{-1}. Unlike the cumulus expansion rate, the *in vitro* maturation rate obtained by the 1,000 ng dose was not significantly ($p> 0.05$) higher than that of positive control group. The *in vitro* maturation rate obtained with this dose was also not significantly different from that of the 100 ng dose. There was no significant difference ($p>0.05$) in the *in vitro* maturation rate between the positive control and the 50 ng dose group.

DISCUSSION

A peptide of 26.6 kDa was partially purified by ammonium sulfate fractionation and molecular exclusion chromatography from buffalo ovarian follicular fluid. Spectrophotometric (absorbance at 280 nm) and SDS-PAGE analysis suggested that the isolated component is a peptide (Ramsoondar et al., 1995).

There have been limited attempts in the past to isolate peptides from the ovarian follicular fluid of buffaloes (Kumar and Pant, 1990; Srivastava and Pant, 1999; Talevi et al., 1999). An inhibin like peptide was isolated by the gel

filtration (Sephadex G-200) of the protein fraction precipitated from the bubaline ovarian follicular fluid by ammonium sulfate saturation at 18.5% level. The peptide inhibited the compensatory ovarian hypertrophy in mice (Kumar and Pant, 1990; Srivastava and Pant, 1999). In this study, follicular fluid protein precipitate obtained by ammonium sulfate precipitation at 30 to 35% saturation level was chosen first, for the isolation of peptides since the quantum of protein precipitate obtained at this saturation level was comparatively larger than at any other level of saturation. In a recent study on follicular fluid peptides in buffalo, ovarian follicular fluid was subjected to ammonium sulfate precipitation at 80% saturation level and the precipitate obtained so was purified through Sephadex G 200. Two minor peaks and one major peak were obtained in the eluant. The partially purified peptide fractions corresponding to the peaks stimulated the progesterone production from buffalo granulosa cells (Mukesh Vinze et al., 2004).

Cumulus expansion is one of the important criteria to assess the *in vitro* maturation rate of oocytes. In the present study the cumulus expansion score was examined to assess the biological effect of different doses of the isolated peptide. Even in cultured but not matured oocytes, there can be some biological effect exerted by the isolated peptide at any level of inclusion in the *in vitro* maturation medium. Thus, in the present study, both cumulus expansion and maturation rates were used to reflect the biological effect of the peptide at different doses.

In the present study, EGF supplemented to the basic *in vitro* maturation medium of oocytes (TCM-199) was considered as the positive control. It was considered as the positive control since EGF supplementation to TCM-199 reportedly yielded more than 80% *in vitro* maturation rates both in sheep (10 ng EGF ml^{-1}) (Guler et al., 2000) and buffalo (20 ng EGF ml^{-1}) (Gupta et al., 2002). Though maturation rate of oocytes is assessed by various methods like staining method (M II stage), degree of cumulus cells expansion (Kobayashi et al., 1994; Chauhan et al., 1997) and identification of extruded first polar body in the perivitteline space; degree of expansion of cumulus cell mass is routinely employed in buffalo IVM for evaluating oocyte maturation for its use in *in vitro* fertilization (Palta and Chauhan, 1998). In conventional *in vitro* fertilization studies, COCs with 70% of homogeneously expanded cumulus cells were considered matured. COCs with slight or no cumulus expansion were discarded though they may have extruded first polar body, thus underestimating the maturation rate. In this study, the COCs with slight or no cumulus expansion but with extruded first polar body were considered matured (Nandi et al., 2002). The high maturation rates obtained by the isolated peptide are comparable to that of the positive control group. In addition, the maturation rates are comparable to that obtained by the usage of conventional maturation media in the same species (Gupta et al., 2001b).

Peptides like IGF, EGF, TGF, FGF and Platelet derived growth factor (PDGF) were reported to be present in the ovarian follicular fluid (Guraya, 1999). Some of these peptides like IGF (Xia et al., 1994; Pawshe et al., 1998), TGFα (Coskun and Lin, 1994), FGF (Gupta et al., 2002), PDGF (Eckert and Niemann, 1996) and EGF (Guler et al., 2000; Gupta et al., 2002) were reported to have stimulated the *in vitro* maturation rate of oocytes in different species of domestic animals. On the other hand TGFβ (Coskun and Lin, 1994), Gonadotropin releasing hormone, Vasoactive intestinal peptide (Beker et al., 2000) and oocyte maturation inhibitor (Dostal et al., 1996) were found to inhibit *in vitro* oocyte maturation rate.

The molecular weight of TGF β1 (25 kDa) (in bovines - Xu-Ruo et al., 1999) is closer to the molecular weight of the isolated peptide, but it inhibits oocyte maturation (Coskun and Lin, 1994) unlike the isolated peptide. Hence, the isolated peptide may not be TGF β1. Further biological characterization of the peptide is possible by wide ranging bioassays using different cell types/animals. Even at the lowest dose of 10 ng ml^{-1}, the isolated peptide could significantly enhance the *in vitro* maturation rate of oocytes. The 50 ng ml^{-1} dose was found to result in *in vitro* maturation rates that were comparable to that of positive control, but were significantly lesser than that obtained with 1,000 ng dose. The maturation rates obtained by 100 ng dose was not significantly different from the highest maturation rates that were obtained with the 1,000 ng dose and hence 100 ng ml^{-1} of maturation medium can be considered as the optimum dose for the incorporation of this peptide in the IVM medium of oocytes in buffaloes.

The peptide with a similar molecular weight that of the isolated peptide in this study was not detected in serum of buffalo (Muralikrishnan, 2002; Personal communication) in other studies conducted at our laboratory. Hence the peptide may be of ovarian origin. Further studies on this peptide at our laboratory have indicated that the peptide could stimulate the *in vitro* maturation rate of oocytes in sheep also which indicates that the peptide is effective in heterologous species too (Gupta et al., 2005). The peptide had also stimulated the folliculogenesis in immature female rats and the effect was reversed by the passive immunization of rats with the antiserum raised against the peptide in rabbit (Communicated). It was also observed that the peptide was present in all the categories of ovarian follicles i.e. small (<6 mm), medium (6-10 mm) and large (11-16) follicles, since the molecular weight and biological property of the peptide from all the categories of follicles was found to be similar (Communicated).

CONCLUSIONS

The results of the present study demonstrated the presence of an oocyte stimulatory peptide of 26.6 kDa in the ovarian follicular fluid of buffaloes, which can be isolated (in a partially purified form) by gel filtration of the protein fraction obtained by saturation (30%-35%) of the ovarian follicular fluid with ammonium sulfate. Further biological and chemical characterization of the peptide would throw more light on its biochemical nature, role and function.

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REFERENCES

Ayoub, M. A. and A. G. Hunter. 1993. Inhibitory effect of bovine follicular fluid on *in vitro* maturation of bovine oocytes. J. Dairy Sci. 76:95-100.

Beker, A. R. C. L., F. Izadyar, B. Colenbrander and M. M. Bevers. 2000. Effect of growth hormone releasing hormone (GHRH) and vasoactive intestinal peptide (VIP) on *in vitro* bovine oocyte maturation. Theriogenology 53:1771-1782.

Bevers, M. M., H. T. M. Van Tol and F. A. M. De Loos. 1992. Protein patterns of immature bovine oocytes incubate with S-methionine in either medium or medium supplemented with dibutyryl-cyclic AMP or follicular fluid. 12th Int. Cong. Anim. Reprod. 1:312-314.

Bruck, I., J. Bezard, G. Duchamp, M. Balsten, P. Dales and T. Greve. 2000. Pure preovulatory follicular fluid for *in vitro* maturation of equine oocytes: An alternative to conventional culture media? Theriogenology 53:450 (Abstr.).

Chauhan, M. S., P. Palta, S. K. Das, P. K. Katiyar and M. L. Madan. 1997. Replacement of serum and hormone additives with follicular fluid in the IVM medium: effects of maturation, fertilization and subsequent development of buffalo oocytes *in vitro*. Theriogenology 48:461-469.

Coskun, S. and Y. C. Lin. 1994. Effects of transforming growth factors and activin A on *in vitro* porcine oocyte maturation. Mol. Reprod. Dev. 38:153-59.

Dostal, J., A. Pavlok and J. E. Flechon. 1996. Isolation and characterization of maturation inhibiting compound in bovine follicular fluid. Reprod. Nutr. Develop. 36:681-690.

Downs, S. M. 1989. Specificity of Epidermal growth factor action on maturation of the murine oocyte and cumulus oophorus *in vitro*. Biol. Reprod. 41:371-379.

Eckert, J. and H. Niemann. 1996. Effects of platelet-derived growth factor (PDGF) on the *in vitro* production of bovine embryos in protein-free media. Theriogenology 46:307-320.

Fujimori, K., R. M. Nakamura, S. A. Tonetta and G. S. Di Zerega. 1987. Cessation of transition-phase follicle growth in the Guinea pig by Follicle Regulatory Protein. Biol. Reprod. 37:812-822.

Guler, A. N., P. Poulin, M. Mermillod, M. Terqui and Y. Cognie. 2000. Effect of growth factors, EGF and IGF-I, and Estradiol on *in vitro* maturation of sheep oocytes. Theriogenology 54:209-218.

Grupen, C. G., H. Nagashima and M. B. Nottle. 1997. Role of epidermal growth factor and insulin-like growth factor-I on porcine oocyte maturation and embryonic development *in vitro*. Reprod. Fertil. Dev. 9:571-575.

Gupta, P. S. P., S. Nandi, B. M. Ravindranatha and P. V. Sarma. 2001a. Effect of buffalo follicular alone and in combination with M199 and PMSG on buffalo oocyte maturation. Asian-Aust. J. Anim. Sci. 14:693-696.

Gupta, P. S. P., S. Nandi, B. M. Ravindranatha and P. V. Sarma. 2001b. Effect of commercially available PMSG on maturation, fertilization and embryo development of buffalo oocytes *in vitro*. Reprod. Fertil. Dev. 13:355-360.

Gupta, P. S. P., S. Nandi, B. M. Ravindranatha and P. V. Sarma. 2002. *In vitro* maturation of buffalo oocytes with Epidermal growth factor and Fibroblast growth factor. Indian J. Anim. Sci. 72:23-26.

Gupta, P. S. P., J. P. Ravindra, V. Girishkumar, H. M. Raghu, S. Nandi. 2005.Stimulation of *in vitro* ovine oocyte maturation with a novel peptide isolated from follicular fluid of the buffalo (*Bubalus bubalis*). Small Rumin. Res. 59(1):33-40.

Guraya, S. S. 1997. Follicular growth and its Regulation and Functions. In Ovarian Biology in Buffaloes and Cattle. 1st Edition, ICAR, New Delhi, India.

Hynes, A. C., M. T. Kane and J. M. Sreenan. 1996. Partial purification from bovine follicular fluid of a factor of low molecular mass with inhibitory effects on the proliferation of bovine granulosa cells *in vitro* and on rat follicular development *in vivo*. J. Reprod. Fertil. 108:185-191.

Harper, K. M. and B. G. Brackett. 1993. Bovine blastocyst development after *in vitro* maturation in a defined medium with epidermal growth factor and low concentrations of gonadotropins. Biol. Reprod. 48:409-416.

Kobayashi, K., S. Yanagamachi and H. Hoshi. 1994: Influence of epidermic growth factor and transforming growth factor-alpha on *in vitro* maturation of cumulus enclosed bovine oocytes in defined medium. J. Reprod. Fertil. 100:439-446.

Kumar, S. and H. C. Pant. 1990. Purification of inhibitor(s) from buffalo follicular fluid to compensatory ovarian hypertrophy in mice. Indian J. Anim. Reprod. 11:135-140.

Laemeli, U. K. 1970. Cleavage of structural protein during the analysis of head of bacteriophage T4. Nature 227:680-685.

Larocca, C., S. Kmaid and J. Calvo. 1993. Effect of follicular fluid and estrus cow serum on maturation, fertilization and development of the bovine oocyte *in vitro*. Theriogenology 39:253 (Abstr.).

Mukesh Vinze, M. K. Sharma and Dheer Singh. 2004. Effect of follicular fluid proteins and gonadotropins on progesterone secretion by buffalo granulosa cells *in vitro*. Asian-Aust. J. Anim. Sci. 17:1496-1500.

Nandi, S., B. M. Ravindranatha, P. S. P. Gupta and P. V. Sarma. 2002. Timing of sequential changes in cumulus cells and first

polar body extrusion during *in vitro* maturation of buffalo oocytes. Theriogenology 57:1151-1159.

Ocana-Quero, J. M., M. Moreno-Millan, M. Pinedo-Merlin and A. Rodero-Franganillo. 1997. Influence of different follicular fractions on *in vitro* maturation of immature bovine oocytes. Arch. de Zootec. 46:51-59.

Palta, P. and M. S. Chauhan. 1998. Laboratory production of buffalo (*Bubalus bubalis*) embryos. Reprod. Fertil. Dev. 10:379-91.

Pawshe, C. H., K. B. C. Appa Rao and S. M. Totey. 1998. Effect of insulin-like growth factor I and its interaction with gonadotrophins on *in vitro* maturation and embryonic development, cell proliferation and biosynthetic activity of cumulus-oocyte complexes and granulosa cells in buffalo Mol. Reprod. Dev. 49:277-89.

Ramsoondar, J., W. Khalil and B. R. Downey. 1995. Partial purification and characterization of a protein in porcine follicular fluid which restricts sperm-egg interaction *in vitro*. Canadian J. Vet. Res. 59:8-14.

Richards, J. S. 1994. Hormonal control of gene expression in the ovary. Endocr. Rev. 15:725-751.

Riley, S. C., R. Thomassen, S. E. Bay, R. Leask, H. G. Pedersen and E. D. Watson. 2004. Matrix metalloproteinase-2 and -9 secretion by the equine ovary during follicular growth and prior to ovulation. Anim. Reprod. Sci. 81:329-339.

Rivera, G. M. and J. E. Fortune. 2003. Selection of the dominant follicle and insulin-like growth factor (IGF)-binding proteins: evidence that pregnancy-associated plasma protein A contributes to proteolysis of IGF-binding protein 5 in bovine follicular fluid. Endocrinology 144:437-446.

Sambrook, J., E. F. Fritsch and T. Maniatis. 1989. Molecular cloning : A laboratory manual, 2nd edn. Cold Spring Harbor, New York.

Srivastava, S. K. and H. C. Pant. 1999. Inhibitory activity in buffalo follicular fluid and its elution pattern during different season. Indian J. Exp. Biol. 37(4):370-373.

Sun, F. J., P. Holm, B. Irvine and R. F. Seamark. 1994. Effect of follicular fluid during *in vitro* maturation of bovine oocytes on *in vitro* fertilization and early embryonic development. Biol. Reprod. 41:981-988.

Tajik, P., S. Goorani-Nejad and H. Ghasemzadeh-Nava. 2000. *In vitro* maturation of water buffalo oocytes in different concentrations of bovine and buffalo follicular fluid. Theriogenology 53: 470 (Abstr.).

Talevi, R., P. Bergamo, G. Sansone, A. Savarese, P. Venditti and P. Abrescia. 1994. Association of cumulus-oocyte complexes with the intrafollicular levels of a blood protein in *Bubalus bubalis.* Zygote 2:167-168.

Xia, P., F. R. Tekpetey and D.T. Armstrong. 1994. Effect of IGF-I on pig oocyte maturation, fertilization, and early embryonic development *in vitro*, and on granulosa and cumulus cell biosynthetic activity. Mol. Reprod. Dev. 38:373-379.

Xu-Ruo, J., Q. C. Doan, G. O. Regester and R. J. Xu. 1999. Detection and characterisation of transforming growth factor-beta in porcine colostrum. Biol. Neonate 75:59-64.

Zak, L. J., X. Xu, R. T. Hardin and G. R. Foxcroft. 1997. Impact of different patterns of feed intake during lactation in the primiparous sow on follicular development and oocyte maturation. J. Reprod. Fertil. 110:99-106.

Zhang, D. and J. Wang. 1994. *In vitro* maturation and fertilization of rabbit oocytes. Acta Agric. Shanghai 10:83-89.