Plant Archives Vol. 20, Supplement 2, 2020 pp. 4245-4250 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

PEPTIDE PROFILE OF BUFFALO OVARIAN FOLLICULAR FLUID

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Abstract

Buffalo (*Bubalus bubalis*) is considered to be less fertile than cattle. Follicular fluid is successfully incorporated as an *in vitro* maturation and fertilization medium in several species. Follicular fluid contains peptide growth factors that are essential for the development of oocytes. These growth factors are either synthesized within the follicle or transported from the blood plasma. The molecular weight of peptide growth factors are < 30 kDa. Buffalo ovarian follicular fluid collected from small, medium and large follicles were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis to resolve < 29 kDa peptides. Thirteen peptide bands of molecular weight 27.3, 25.6, 22.5, 19.6, 18.2, 16.8, 15.9, 14.7, 14, 12, 8.3, 5.5 and 3.7 kDa were resolved and the electrophoretic pattern was found to be similar among different sized follicles. The relative quantity (%) of these bands didn't vary significantly (P>0.05) between different sized follicle. Total number of bands and electrophoretic pattern was similar between follicle and serum. Comparison with reports in other species revealed the presence of low molecular weight peptide growth factors such as insulin like growth factors, epidermal growth factors, fibroblast growth factors, insulin like growth factor- binding proteins, oocyte stimulatory peptide, inhibin and haptoglobin in buffalo ovarian follicular fluid.

Keywords: Follicular fluid, peptides, growth factors, buffalo, SDS- PAGE.

Introduction

Follicular fluid (FF) provides a vital diminutive environment for the development of oocytes. The quality of oocyte and its ability to undergo fertilization and embryo development is decided by the FF biochemical milieu. FF contains essential peptides that are synthesized *in situ* within the follicle by granulosa and theca cells (Webb *et al.,* 2007) and it also comprises plasma proteins that are transferred through blood follicle barrier (Hess *et al.,* 1998).

Growth factors (GFs) are ubiquitous peptides that regulate the survival, proliferation and differentiation of follicular cells (Monniaux *et al.,* 1997). By paracrine and/or endocrine actions GFs modulate folliculogenesis, steroidogenesis, oocyte maturation, ovulation and corpus luteum function. Peptide GFs present in the follicle decides the fate of ovum by interaction with gonadotropins and gonadal steroids (Matsuda *et al.,*2012). The molecular weight (MW) of peptide GFs are reported to be <30 kDa (Hill, 1989). Some of the high MW proteins undergo fragmentation to yield peptides which retains biological activity are called cryptides (Pimenta and Laeburn, 2007).

Peptide GFs that are involved in folliculogenesis are Insulin like growth factors (IGF), Epidermal growth factor (EGF), Fibroblast growth factor (FGF), Platelet derived growth factor (PDGF), Transforming growth factor β (TGF –β), Transforming growth factor α (TGF –α), Platelet-derived growth factor (PDGF), Tumor necrosis factor (TNF), Hepatocyte growth factor (HGF), Nerve growth factor (NGF), Vascular endothelial growth factor (VEGF), Vasoactive intestinal peptide (VIP), inhibin and activin system (Kumar *et al*., 2014).

Assisted reproductive technology (ART) protocols are widely bestowed in livestock to preserve and to improve germplasm (Romar *et al.,* 2015). FF has been widely incorporated as *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) medium because of its greater ability to stimulate nuclear and cytoplasmic maturation of oocyte, fertilization and development of embryos. This ability was due to the presence of various stimulatory and inhibitory peptide growth factors (Tsafriri and Adashi, 1994). FF was successfully tested as IVM and IVF medium in several species such as cattle (Satitmanwiwat *et al.,* 2017), horse (Gil *et al.,* 2005), sheep (Guler *et al.,* 2000) and pig (Ferre *et al.,* 2015). The ART protocol is not much successful in buffaloes (*Bubalus bubalis*) due to its inherently low fertility and its poor response to superovulation. In buffaloes, poor oocyte maturation and low cleavage rate leads to failure of *in vitro* embryonic production. The overall efficiency of *in vitro* production of embryos is much lower in buffaloes than in cattle and the average yield of transferable embryos is less than one per super ovulated donor (Warriach *et al.,* 2015).

Studies on low molecular weight peptide profile and growth factors in buffalo follicular fluid (bFF) are meagre. To fill the gap, this study was taken up to know the low molecular weight peptide profile (< 29kDa) of FF during its different developmental stage. Identification of any developmental stage specific peptide in FF would be a biomarker of folliculogenesis in buffaloes.

In this study we used Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS- PAGE) with high concentration of separating gel to resolve low molecular weight peptides.

We have compared the MW of the identified peptides with the earlier reports in other species to speculate its identity.

Materials and Methods

Collection and transportation of ovaries

Ovaries from adult, healthy buffaloes of unknown reproductive status were collected immediately after slaughter in civil abattoir, Bangalore. Collected ovaries were transported within an hour to laboratory in plastic bags containing chilled 0.9 % sodium chloride and gentamicin 50 µg/ml. Sixteen to twenty ovaries were collected each week for twenty weeks (n=382).

Classification of follicles, collection and storage of follicular fluid

Ovaries were washed thoroughly with chilled normal saline in the laboratory. A hand lens and scale of 1mm sensitivity was used to measure follicle diameter. On the basis of the surface diameter (Kulkarni, 1988), follicles on the ovary were grouped as SF (< 6 mm), MF (6 to 10 mm) and LF (11 to 16 mm). Tuberculin syringe with 26 gauge needle was used for aspiration of the FF from SF and 5ml disposable syringe with 22 gauge needle was used for aspiration of FF from MF and LF. FF obtained from SF, MF and LF of all ovaries in a collection were pooled separately. The pooled FF from three different sized follicles were centrifuged separately at 6000 rpm for 30 minutes at 5^0C to remove cellular debris and supernatant was collected. To prevent proteolysis Phenyl methyl sulfonyl fluoride (PMSF) at the rate of 20 µg/ml of FF was added and samples were stored at -20 ºC.

Collection and processing of serum samples

Blood samples were collected in a sterile glass tubes from buffaloes immediately after slaughter and transported to laboratory at 5^0C in aseptic condition. Serum was separated and centrifuged at 3000 rpm for 15 minutes under refrigerated condition (5^0C) to remove cellular debris. The supernatant was collected and PMSF at the rate of 20 µg/ml of serum was added and stored at -20^0C. Serum samples collected each week were pooled as single sample.

SDS-PAGE fractionation of follicular fluid and serum peptides and/ or proteins

The total protein concentration (TP) of FF and serum was estimated (Krishnan *et al*., 2005) as per standard procedures. SDS-PAGE was carried out under reducing condition as per the methods of Laemmli, (1970). Vertical slab gel measuring 160 mm × 140 mm was used with 4.5% stacking gel containing 0.5M Tris-Hcl at pH 6.8 and 12.5% resolving gel containing 1.5M Tris-Hcl at pH 8.8. The protein molecular weight marker containing ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soyabean trypsin inhibitor (20 kDa), lysozyme (14.3 kDa), aprotinin (6.5 kDa), insulin (3 kDa) were used as standard.

FF samples of SF, MF and LF with a TP of 400 µg and serum samples with 100 µg of total protein were diluted in10 µl of sample buffer (0.5 M Tris-Hcl, 10% SDS, 50% glycerol, β mercaptoethanol, pH 6.8,) and loaded along with 20µl of 1% bromophenol blue in separate lanes. Running buffer used has 250 Mm Tris, 1.92M glycerine, 1% SDS with pH 8.3. Electrophoresis was carried out with a constant voltage of 100 V at room temperature for 8 hrs until the tracking dye reaches 1 cm before the bottom. Gels were washed and stained with 0.25 % Coomassie brilliant blue R-250 prepared with 40% methanol and 10% acetic acid for 10 hrs. Destaining was completed with solution containing 40% methanol and 10% acetic acid. SDS PAGE was repeated with twenty different samples of pooled FF from SF, MF, LF and sixteen samples of serum.

Molecular weight and Relative quantity (RQ) of peptide band

Detection of band, analysis of its MW and RQ(%) was done by scanning the electrophoretic gels (n=20) in Gel Documentation System using Quantity One 1-D analysis software (Bio-Rad, USA). Data analysis was carried out by ANOVA using SPSS software.

Results and Discussion

SDS-PAGE profile of low molecular weight peptides

Although our study was conducted with basic 1D -SDS PAGE technique it revealed the existence of several low MW peptides in buffalo ovarian FF which was not reported earlier. Various low MW peptides resolved by 12.5% SDS PAGE is shown in Image I. Thirteen bands were observed in FF of SF, MF and LF indicating similar electrophoretic pattern among different sized follicles. Total number of bands and electrophoretic pattern was found to be similar between FF and serum.

Relative quantity of each peptide bands

The MW and RQ (%) of 13 peptide bands in different sized follicles are presented in Table I. The RQ (%) of these bands were found not to vary significantly ($P > 0.05$) between different sized follicle. The 27.3 kDa MW was found to be a major band followed by 25.6 kDa, 22.5kDa and 14.7 kDa. All other peptide bands are minor bands. Major and minor bands in serum was similar to FF.

Since we used SDS PAGE under reducing condition, the peptides identified in this study is not intact protein, they are either single chain polypeptides or individual subunits of a native protein. Each band may contain either single peptide or may have more than one peptide of nearly similar MW. Based on these criteria a comparison of MW was made with peptides reported in the previous studies to speculate its identity (Table II).

Some of the proteins are synthesized predominantly in the follicle such as inhibin and eventually enters into the circulation for their endocrine action (Findlay, 1993). On the other hand, growth factors such as IGFs, EGF, and FGF are synthesized locally in follicle as well as elsewhere in the body (Monget and Monniaux, 1995). The similarity in the electrophoretic pattern between the FF and serum observed in the present study could be because these proteins and/or peptides are not restricted to the place were they are synthesized.

The peptide profile of our study is similar during the different developmental stage and it agrees with Fahimiya *et al.*(2011) who reported that protein profile of FF collected during three physiological stages revealed no differential proteins. Rocha *et al.* (2015) reported that it was not possible to correlate a specific protein with a particular stage of follicular development in mare FF. Similarly, Ducolomb *et al.* (2013) also reported that in porcine FF there is no connection between specific proteins with a particular stage of oocyte maturation.

Inhibin acts as a negative feedback hormones that regulates the synthesis and secretion of pituitary FSH and indirectly stimulate follicle development (Knight *et al*., 2012). Among 13 peptide bands resolved , we suspect nearly 10 peptides which are below 30 kDa to be subunits of inhibin. Presence of multiple molecular forms of inhibin and subunits upto 20 kDa was reported in buffaloes by Ganguly *et al.* (2010). We suspect presence of inhibin subunits upto 5.5 kDa in bFF as there are reports in other species that inhibin subunits are present upto 6 kDa.

The function of IGF is that it acts in synergistic with gonadotropins to promote growth and steroidogenesis of ovarian cells. Actions of IGF I and II are controlled by IGF binding proteins (IGFBP) that are transferred from the blood or are synthesized locally within the follicle. The action of low MW IGFBPs (IGFBP-2, -4, -5) are to bind with IGF and decrease its bioavailability. IGFBP is found in traces in dominant and preovulatory follicle whereas subordinate follicles exhibit higher level (Lucy, 2000). Three peptide bands of MW between 27 and 22 kDa reported here may be IGFBP.

The 27.3 kDa peptide observed in the present study was same as the 26.6 kD Oocyte stimulatory peptide isolated from buffallo FF and found to have stimulatory effect on *in vitro* cumulus expansion and *in vitro* maturation rate of oocytes in sheep and buffaloes (Gupta *et al*., 2005).

FGF plays a vital role in supporting the growth and development of the granulosa-luteal cell. Misra *et al.* (2016) has reported the presence of FGF in buffalo follicle during different stages of development that promote steroidogenesis and granulosa cell survival through autocrine and paracrine action. We suspect the 16.8 kDa peptide may be FGF as the MW of FGF is 16-17 kDa as per the reports of Hill (1989).

EGF plays an important role in finely regulating oocyte maturation and ovulation (Richani and Gilchrist, 2018). EGF-like growth factors accumulate in the follicle during ovulation. The EGF-like growth factors amphiregulin, epiregulin, and betacellulin are potent stimulators of oocyte maturation and cumulus expansion, and perturbation of this EGF network in vivo impairs ovulation. (Hsieh *et al*., 2009). The 5.5 kDa peptide identified in this study may be EGF.

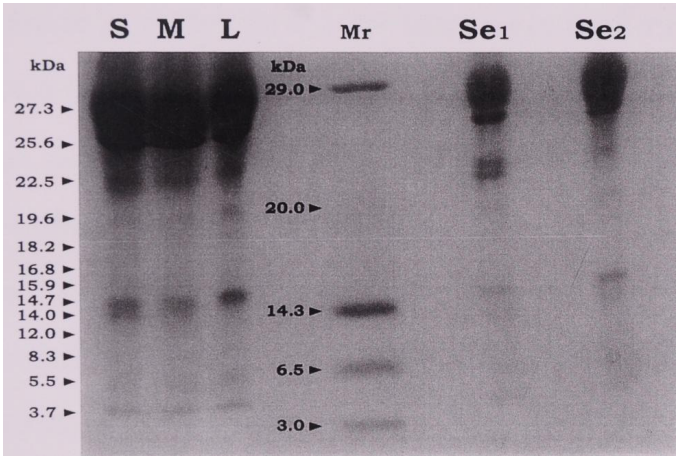
Haptoglobin-like protein is a normal constituent of bovine ovarian and oviductal tissues and fluids that contributes to ovarian follicular development and oviductal function (Lavery *et al*., 2003). In water buffalo, haptoglobin is used as a molecular marker to assess the physiological state of the blood-follicle barrier or to differentiate between atretic and healthy follicles (Bergamo *et al*., 1995). The 22.5 and 15.9 kDa peptide could be subunits of haptoglobin.

Our results proved that in the era of 2D PAGE, studies using 1D-SDS PAGE is promising to resolve low molecular peptides. Earlier studies in buffaloes have reported the presence of peptides of MW, only up to 20 kDa and peptides below 20 kDa have not been reported. Although we compared the MW of peptide band resolved in this study with the earlier reports, in future these peptides should be isolated, characterized and its biological activity has to be assessed to confirm its identity and its role in folliculogenesis.

Conclusion

Buffalo ovarian follicular fluid contains < 29 kDa molecular weight peptides that may be growth factors. Thirteen peptide bands of molecular weight 27.3, 25.6, 22.5, 19.6, 18.2, 16.8, 15.9, 14.7, 14, 12, 8.3, 5.5 and 3.7 kDa were resolved and the electrophoretic pattern was similar between different sized follicles and serum. Further studies on isolation and characterization of each peptide would reveal its identity and its importance in folliculogenesis.

Image 1: SDS-PAGE profile of buffalo ovarian follicular fluid peptides



(**Note**: FF collected from S = small, M = medium, L = large follicle, Se1 and Se2 = serum and Mr = standard molecular weight marker)

Table I: Molecular weight of peptide bands and their relative quantity (%) in different sized follicles

Band	Molecular weight (kDa)	Small follicle RQ (%)	Medium follicle RQ(%)	Large follicle RQ(%)
1	27.3 ± 0.11	39.99 ± 1.27	39.73 ± 1.10	39.04 ± 1.84
2	25.6 ± 0.07	23.80 ± 1.04	24.69 ± 0.99	24.70 ± 1.46
3	22.5 ± 0.09	9.79 ± 0.85	8.44 ± 0.76	9.16 ± 0.71
4	19.6 ± 0.09	1.00 ± 0.17	1.11 ± 0.20	1.68 ± 0.23
5	18.2 ± 0.10	0.56 ± 0.14	0.68 ± 0.09	0.85 ± 0.12
6	16.8 ± 0.98	0.15 ± 0.02	0.27 ± 0.03	0.23 ± 0.02
7	15.9 ± 0.04	1.10 ± 0.11	0.90 ± 0.12	0.83 ± 0.13
8	14.7 ± 0.09	7.41 ± 0.44	7.34 ± 0.26	6.46 ± 0.39
9	14.0 ± 0.16	3.38 ± 0.24	3.80 ± 0.43	3.83 ± 0.56
10	12.0 ± 0.16	1.99 ± 0.23	2.44 ± 0.41	2.31 ± 0.37
11	8.3 ± 0.13	2.15 ± 0.23	2.28 ± 0.19	1.78 ± 0.19
12	5.5 ± 0.04	4.24 ± 0.42	4.03 ± 0.34	4.46 ± 0.40
13	3.7 ± 0.03	4.56 ± 0.42	4.42 ± 0.62	4.72 ± 0.50

Note: All values of MW are mean ± SEM and values of RQ of different sized follicles are mean ± SEM (%). RQ of 13 peptide bands were found not to differ significantly (P>0.05) between different sized follicles.

Table II: Comparison of molecular weight of peptide bands identified in the present study with previous reports in different species

MW of Peptide Band	MW of peptide band reported earlier	Species	Sample	Name of the Peptide	Reference
27.3	29	Buffalo	FF	Inhibin subunit	Ganguly *et al*. (2010)
	26.6	Buffalo	FF	Oocyte stimulatory peptide	Gupta *et al*.(2005)
	27-29	Bovine	FF	IGFBP-5	Chamberlain and Spicer (2001)
25.6	25	Buffalo	Plasma	L-chain of Ig	Kulkarni *et al*.(1998)
	25	Buffalo	Serum	L-chain of IgG	Ratyal *et al*. (1992)
	25	Bovine	FF	Inhibin subunit	Knight *et al*. (1989)
	24	Bovine	FF	IGFBP-4	Rivera and Fortune (2001)
	21-24	Bovine	Serum	IGFBP-3	Twigg *et al*. (2000)
22.5	22	Bovine	Follicle	IGFBP-4	Chamberlain and Spicer (2001)
	22	Bovine	FF	α subunit -InhibinA	Silva *et al*.(1999)
	21	Buffalo	FF & serum	α subunit of haptoglobin	Bergamo *et al*.(1995)
19.6	20	Buffalo	FF	Inhibin subunit	Ganguly *et al*. (2010)
	20	Bovine	Granulosa cell	Inhibin subunit	Boudjemaa *et al*. (2000)
18.2	18	Porcine	FF	Inhibin subunit	Ling *et al*. (1985)
16.8	16-17	Bovine	Pituitary	FGF	Hill,1989
15.9	16	Bovine	Plasma	Subunit of bovine haptoglobin	Eckersall and Conner (1990)
	16	Ovine	FF	Inhibin subunit	Leversha *et al.* (1987)
14.7	15	Bovine	Granulosa cell	Inhibin subunit	Boudjemaa *et al*. (2000)
	15	Bovine	FF	Inhibin/ Activin β A subunit (monomer)	Robertson *et al*.(1992)
14	14	Bovine	FF	Inhibin subunit	Knight *et al*. (1990)
12	13	Bovine	FF	Inhibin subunit	Fukuda *et al.* (1986)
8.3	7.5	Bovine	Fetal bovine serum	IGF	Valenzano *et al*. (1997)
5.5	6	Bovine	Follicle	Inhibin subunit	Sugino *et al*. (1989)
	6	Bovine		EGF	Kumar *et al*.(2014)
3.7	< 5	Bovine	FF	Granulosa cell inhibiting factor	Hynes *et al*. (1996)
	< 5	Ovine and human	Granulosa cells	Ovarian follicular fluid peptide (OFFP)	Nandedkar *et al*. (1996)
	1-3	Porcine	FF	Low molecular weight inhibitory substance	Kigawa *et al.* (1986)
	>1 & < 6.5	Porcine	FF	Low molecular weight heat stable factor	Daen *et al*. (1995)

Acknowledgement

The author expresses his gratitude to Dr. V. Girish Kumar, Professor and Head, Department of Veterinary Biochemistry, Veterinary college, Bangalore, KVAFSU, Karnataka for his guidance and support.

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