Plant Archives Vol. 20 Supplement 1, 2020 pp. 1849-1852 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

IDENTIFICATION OF HIGH MOLECULAR WEIGHT PROTEINS IN BUFFALO (*BUBALUS BUBALIS*) OVARIAN FOLLICULAR FLUID BY SDS PAGE

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Abstract

Buffalo contributes to agricultural economy by providing milk and meat for sustainable food production. In spite of its milk production ability, the reproductive efficiency is low in buffaloes. Follicular fluid was successfully tested as *in vitro* maturation and fertilization medium in several species as it contains stimulatory and inhibitory proteins. Buffalo follicular fluid alone without any supplementation induced good maturation of buffalo oocytes. Ovarian follicular fluid from different sized follicles were collected from buffalo ovaries obtained from slaughter house. To know the protein profile, follicular fluid and serum was subjected to SDS PAGE and 25 bands of MW[3] 29 kDa was resolved. A similar electrophoretic pattern was observed in small, medium and large follicles as well as in serum. Haptoglobin, buffalo serum albumin, inhibin subunits and other unknown proteins/peptides were identified.

Key words: Buffalo, Follicular fluid, SDS PAGE, Protein**.**

Introduction

Buffalo (*Bubalus bubalis*) contributes significantly to agriculture sector for sustainable food production. In India and in South Asian countries, it serves as a source of milk, meat and draught. In spite of being major contributor to dairy industry, they are highly susceptible to reproductive disorders and are known for its poor reproductive ability. Reproductive disorders such as delay in onset of puberty, poor estrus expression, longer duration of postpartum ovarian quiescence and low conception rates are common in buffaloes (Warriach *et al*., 2015). Poor fertility in buffalo is associated with less primordial and antral follicle development, a slower shift from small to large follicle, higher incidence of follicular atresia, anovulation (Manik *et al*., 2002) and anestrous condition (Kumar *et al*., 2019). Better understanding of mechanisms involving follicular growth and development is essential for improving breeding efficiency of buffaloes (Singh *et al*., 2019).

Ovarian follicular fluid (FF) plays a major role in the physiological, biochemical and metabolic aspects of the nuclear and cytoplasmic maturation of the oocyte, release of the egg from the ruptured follicle, and sperm hyperactivation. It is not a simple transudate of blood, but it is a complex of restricted components of serum and follicular synthesized secretions (Gosden *et al*., 1988). Some of the proteins present in follicle influence follicular development and oocyte maturation. Folliculogenesis is regulated by an interplay of extraovarian and intraovarian factors. FF when used as *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) medium was found to have greater ability to stimulate nuclear and cytoplasmic maturation of oocyte, *in vitro* fertilization and development of embryos (Satitmanwiwat, 2017). Buffalo FF alone without any supplementation induced good maturation of buffalo oocytes (Gupta *et al*., 2001). This positive response was because of the presence of various

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stimulatory proteins/peptides present in FF.

The identification and role of buffalo FF peptides/ proteins have not been elucidated completely. Hence the present study is an attempt to identify various high molecular weight proteins in FF, among which some of them may play a role in follicular growth and regression.

Materials and methods

Buffalo ovaries were collected from healthy animals of unknown reproductive status immediately after slaughter and evisceration from local civil slaughter house, Bangalore. In each collection 50 ovaries were collected and collection was done for 20 weeks. Smooth ovaries without any follicles were discarded and ovaries bearing good number follicles were selected for further processing. Following collection, ovaries were washed twice with sterile 0.9 percent NaCl (w/v) and placed in sterile plastic bag containing ice cold 0.9 percent NaCl and transported to the laboratory within one hour. On the basis of the surface diameter (Kulkarni, 1988), all the follicles on the ovary were grouped as SF (< 6 mm), MF (between 6 and 10 mm) and LF (between 11 and 16 mm). FF was collected from all the three different sized follicles separately by aspiration. The FF from three different sized follicles were centrifuged separately in a cooling centrifuge (Remi make: model C-23) at 6000 rpm at 5^0C for 30 minutes to remove the blood cells, oocyte and granulosa cells. Phenyl methyl sulfonyl fluoride (PMSF) at the rate of 20 mg/ml was added to the cell free FF to prevent proteolysis during storage. The cell free FF samples were stored at -20^0C until used for further analysis.

Blood samples were also collected aseptically into glass tubes at the time of slaughter from those buffaloes in which ovaries were obtained. Serum was separated and centrifuged at 3000 rpm for 15 minutes under refrigerated condition (5^0C), to remove blood cells as soon as possible. PMSF at the rate of 20 mg/ml serum was added and stored at -20^0C for further analysis.

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

The total protein concentration of FF and serum was estimated (Krishnan *et al*., 2005). 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 7.5% resolving gel containing 1.5M Tris-Hcl at pH 8.8. The protein molecular weight marker containing myosin, rabbit muscle (205 kDa), Phosphorylase b (97.4 kDa), Bovine serum albumin (66 kDa), Ovalbumin (43 kDa) and Carbonic anhydrase (29 kDa) were used as standard.

FF samples of SF, MF and LF with a TP of 100µg and serum samples with 100µg 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 1cm 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 (MW) of band

In order to identify total number of bands present and to know its MW, electrophoretic gels (n=20) containing resolved bands were scanned and analyzed in Gel Documentation System using Quantity One 1-D analysis software (Bio-Rad, USA).

Results and Discussion

The electrophoretic pattern of peptides (MW ≥ 29 kDa) in FF of SF, MF, LF and serum is shown in plate 1. The mean molecular weight for each band (MW ≥ 29 kDa) of the replicates irrespective of size of follicle is shown in table 1. Total number of bands observed in FF was 25. All the 25 bands were present in the FF of SF, MF, and LF indicating similar electrophoretic pattern among different sized follicles. The total number of bands and electrophoretic pattern was similar in serum and FF.

Electrophoretic pattern in different sized follicles

In the present study, 25 bands were identified in FF whose molecular weight (MW) was ≥ 29 kDa when 7.5 percent gel was used. Likewise, Kulkarni (1988) reported the presence of 2, 6 and 18 bands of MW < 25, > 116 and 25-116 kDa, respectively using 10 percent gel by SDS-PAGE. Joy *et al.,* (2015) observed 34 bands in buffalo follicular fluid and serum. The difference in the number of bands observed in the earlier studies is due to difference in the percentage of resolving gel used.

Among the 25 bands of MW were ≥ 29 kDa, the 246 kDa protein may be haptoglobin (Bergamo *et al*., 1995). The 69.9 kDa peptide was suspected to be buffalo serum albumin whose MW was reported as 69 kDa (Tayyab and Qasim, 1990). Likewise, the 42.9 kDa peptide may

be a form of inhibin subunit as identified in bovine FF (Findlay *et al*., 1994). The 33.4 kDa band corresponds to 34 kDa inhibin form (Draincourt *et al*., 2001). A 30 kDa unknown peptide isolated from buffalo follicular fluid delayed the onset of estrus in ewes and increases ovulation rate (Ghosh *et al*., 2005). Earlier study confirms that the 29 kDa peptide is an inhibin subunit (Ganguly *et al*., 2010).

Electrophoretic pattern of peptides/proteins (MW ≥ 29 kDa) in follicular fluid and serum

In the present study, the SDS-PAGE pattern was similar when compared between SF, MF, LF and serum. This observation is same as that of Kulkarni, (1988), who reported a similar SDS-PAGE pattern between different sized follicles and serum of buffalo. Similarly, studies using agar gel, polyacrylamide gel and immuno-electrophoresis showed that the electrophoretic pattern between FF and serum was identical in goat (Sidhu *et al*, 1985) and buffalo (Parmer and Metha, 1991). Likewise, Khan *et al*., (2013) reported that there was no apparent difference in SDS PAGE pattern between follicular fluid from small, medium and large follicles of cyclic and acyclic buffaloes.

Molecular weight of protein/peptide bands (Mean ± SEM).

Band	Molecular weight (kDa)
1	246.0 ± 0.23
2	226.7 ± 1.59
3	208.4 ± 0.21
4	164.1 ± 0.17
5	137.2 ± 0.20
6	129.5 ± 0.42
7	120.9 ± 0.11
8	115.5 ± 0.38
9	101.6 ± 0.38
10	95.9 ± 0.14
11	92.7 ± 0.14
12	85.3 ± 0.08
13	81.6 ± 0.16
14	76.9 ± 0.05
15	73.6 ± 0.09
16	69.9 ± 0.23
17	56.1 ± 0.05
18	49.1 ± 0.18
19	42.9 ± 0.06
20	38.8 ± 0.07
21	37.1 ± 0.08
22	33.4 ± 0.03
23	31.1 ± 0.08
24	30.0 ± 0.02

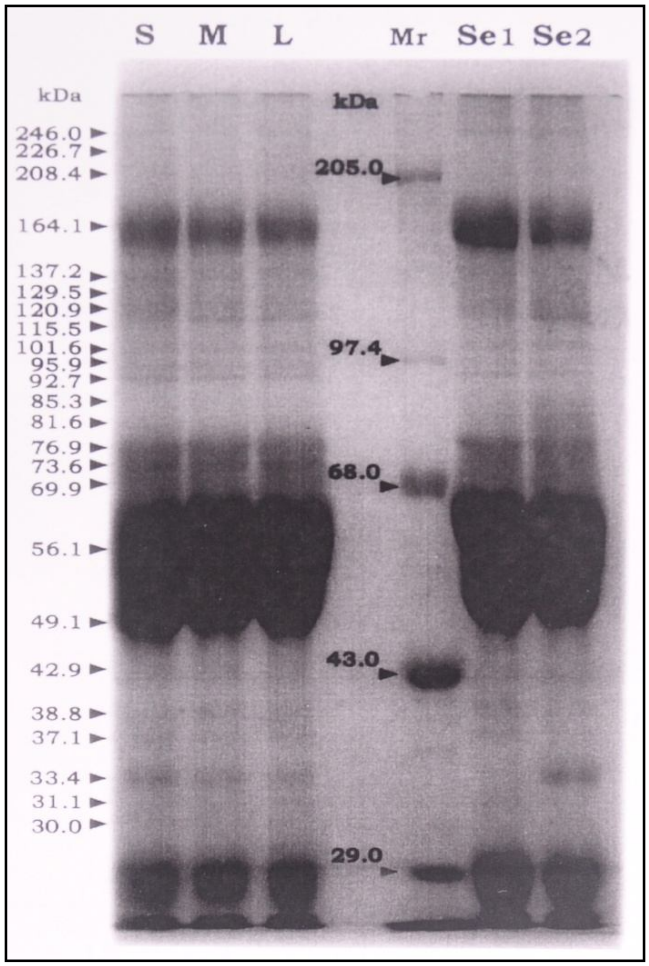
Parmer and metha (1992) observed a strong antigenic cross reactions between FF and plasma proteins indicating most of the FF and plasma proteins to be structurally and functionally similar. This could be the reason for similar electrophoretic pattern in SF, MF, LF and serum observed in the present study.

Further, it was found that the proteins present in the FF especially, albumin, globulin, and steroid binding proteins were mainly derived from the plasma and were not locally synthesized in the follicle (Andersen *et al*., 1976). Whereas, some proteins and /or peptides may be 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 (Kumar *et al*., 2014). The similarity in the electrophoretic pattern between the FF and serum observed in the present study could be because these high molecular proteins are not restricted to the place were they are synthesized and are freely permeable between follicle and blood.

Conclusion

The bands identified in this study may be an indvidual protein or a subunit of a protein. Future research on isolation and testing the biological activity of individual protein is essential to confirm its identity. Individual proteins thus isolated may be tested by supplementation in IVM and IVF medium. Better understanding of the role of various stimulatory and inhibitory proteins in folliculogenesis and regression is essential to augment the reproductive efficiency of buffaloes.

SDS-PAGE Profile of buffalo ovarian follicular fluid and serum proteins/peptides of MW ≥ 29 kDa



Note: S = Small follicle, M = Middle sized follicle, L = Large follicle Mr = Molecular weight marker, Se1 and Se2 are serum samples.

Acknowledgement

The author is thankful to Dr. V. Girish Kumar, Professor and Head, Department of Biochemistry, Veterinary College, Bangalore for his guidance and support.

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