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SEASONAL INFLUENCE ON QUANTITATIVE PROFILING OF PROTEINS IN BUFFALO (*BUBALUS BUBALIS*) OVARIAN FOLLICULAR FLUID

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Abstract

Buffaloes (*Bubalus bubalis*) provide nutrition and livelihood security to rural agricultural community. They are seasonal breeders and its reproductive efficiency varies with season. Breeding activity is negatively related to photoperiod and its fertility increases with decrease in day length. To study its seasonality, buffalo ovaries were collected during its breeding season (monsoon and winter season) and follicular fluid (FF)was aspirated from small (SF), medium (MF) and large sized follicles (LF).FF proteins were separated by SDS PAGE (n=20). The relative quantity (RQ) of each band was analyzed by Gel Documentation System and was compared between season and between different sized follicles. Total number of bands of molecular weight (MW)> 29 kDa were 24 and all the bands were present during monsoon, winter season and in different sized follicles. Among these 24 bands the RQ of only one protein band of MW 37.1kDa significantly decreased ($P<0.05$) with increase in size of the follicle. Comparison of 37.1 kDa band with bovine FF implies that it may be follistatin. There was a significant increase ($P<0.05$) in the RQ of 226.7, 120.9 and 95.9 kDa bands during monsoon compared to winter season. The 120.9 kDa band identified may be a form of IGFBP .Out of 24 protein bands, 21 bands did not vary significantly within its breeding season. Our results imply that within the breeding season buffalo FF contains qualitative and quantitively similar proteins essential for the growth and maturation of ovarian follicles and oocyte.
***Keywords*: Season, buffalo, follicular fluid, protein, SDS-PAGE.**

Introduction

Buffaloes (*Bubalus bubalis*) play an important role in enhancing rural agricultural economy. They occupy a pivotal place both in the farming systems and in the nutrition and livelihood security of rural families. In India, more than 55 % of total milk production is from buffaloes. In spite of being a major contributor to the dairy industry, their reproductive efficiency is poor. Late sexual maturity, long postpartum anoestrus, poor expression of oestrus, poor conception rates and long calving intervals are common in buffaloes (Perera, 2011). One of the main reasons is that it is a seasonal breeder and its breeding activity is negatively related with photoperiod. Its fertility increases with decrease in daylength. Climate has an influence on fertilization, implantation, growth of the fetus as well as on hormonal and biochemical balance (D'Occhio *et al.,* 2020). Anestrus is common in buffaloes during summer season (Das and Khan, 2010). The highest ovarian activity including oocyte recovery rate, oocyte quality and oocyte maturation rate was found during winter and spring in Egyptian cattle and buffaloes (Soliman *et al.,* 2016).

Seasonal variation in fertility of buffaloes causes huge economic loss to farmers. In order to improve the reproductive efficiency of buffaloes during its non breeding season, it is imperative to understand the influence of season on its follicular dynamics (Singh *et al*., 2019). This study was taken up to know if there are any season specific proteins in the follicle and any variation in its quantity during different stages of development in buffaloes.

Materials and Methods

Ovaries from buffaloes were collected during monsoon (September, October and November) and winter season (December and January) from healthy animals immediately after slaughter and evisceration in civil slaughter house, Bengaluru. Fifty ovaries were collected during each week and was immediately transferred to the laboratory. 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 and were pooled according to its size. The pooled FF from three different sized follicles were centrifuged 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 and stored at -20^{0}C. The total protein concentration of FF in different sized follicles were also estimated (Bradford, 1976).

The pooled FF from three different sized follicles were subjected to SDS-PAGE (n=20) under reducing condition as per the method of Laemmli (1970). Stacking gel of 4.5% and resolving gel of 7.5% was used. Same quantity (100 µg) of FF samples of SF, MF and LF was loaded in each well. Electrophoretic gels were scanned and analyzed in gel documentation system to know the MW and RQ(%) of each band using quantity one 1-D analysis software (Bio-Rad). The data was analysed statistically using Graph pad prism software. The RQ of band was compared between different sized follicles (irrespective of season) by One way analysis of variance (ANOVA) followed by a post test called Bonferroni's multiple comparison test and between seasons (irrespective of follicle size) by students 't' test.

Results and Discussion

The mean RQ (%) of each band in different sized follicles and during monsoon and winter season are shown in table 1. The total number of electrophoretic bands of MW > 29 kDa was 24 and all thebands were present in different sized follicles. Electrophoretic protein profile was similar in different sized follicles (Krishnan *et al*., 2020). Earlier report indicates that total protein concentration is similar in different sized follicles in buffaloes (Krishnan *et al.*, 2005; Satheshkumar *et al.*, 2016). Similarly, the RQ (%) of electrophoretic protein bands (irrespective of season) did not vary significantly (P >0.05) between different sized follicles except in one band. The RQ of 37.1 kDa band significantly decreased (P<0.05) with increase in size of the follicle. The 37.1 kDa band observed in the present study is comparable with follistatin isoform of MW 37 kDa reported by Glister *et al.* (2006) in bovine follicular fluid. He also reported that follicle growth from 9 to 20 mm resulted in highly significant 2-fold decrease in follistatin concentration. Similarly, Ferraza *et al.*,(2017) reported a high concentration of follistatin in early stage of development of bovine follicle. Follistatin plays a negative role on follicular growth and function by acting either directly or indirectly by suppressing the active in (Glister *et al.*, 2015). The 33.4 and 31.1 kDa observed in this study is also comparable to the follistatin isoforms of molecular weight 33 kDa and 31 kDa (Glister *et al.,* 2015). The 33.4 and 31.1 kDa band did not vary significantly in different sized follicles.

Seasonal variations in breeding activity is more common in buffaloes (Phogat *et al.,* 2016). Breeding season coincides with the months of the year with low environmental temperature and decrease in day length. In India, the period between September to February is a favourable breeding season for buffaloes (Hegde *et al.*, 2019). Previous studies reported variations in concentration of hormones within a breeding season. In buffaloes, Sheth *et al.* (1978) reported a higher LH concentration in monsoon compared to winter season. Sharma *et al.*, (2014) observed the presence of season specific seminal plasma proteins in bhadawari bulls. Gunwant *et al.,* (2018) concluded that buffaloes have a tendency to mate and calve more in the days with shorter photoperiod as compared to days with longer photoperiod. He reported that maximum calving was during the month of September and October. Maximum percentage of buffaloes exhibit estrous in the month of November and December. Dutra *et al.* (2019) reported season specific proteins in equine follicular fluid. In the present study the total number of electrophoretic bands remains same in monsoon and winter season. The RQ(%) of bands (irrespective of its follicle size) did not vary significantly (P >0.05) between monsoon and winter season, except for three bands. There was a significant increase (P<0.05) in the RQ of 226.7, 120.9 and 95.9 kDa bands in monsoon compared to that in winter season. The 120.9 kDa band is comparable with 120 kDa IGFBP identified in equine follicular fluid (Gerard and Monget, 1998). The 226.7 and 95.9 kDa bands may be subunits of FF proteins. Studies shows that the total protein concentration in buffalo ovarian FF did not vary significantly between monsoon and winter season (Krishnan *et al.*, 2005). Similarly, quantitative protein profiling revealed that among 24 protein bands the majority of proteins (21 bands) did not vary significantly between monsoon and winter season. Our results imply that within the breeding season buffalo FF contains qualitative and quantitively similar proteins essential for the growth and maturation of ovarian follicles and oocyte.

Conclusion

Our study revealed that follistatin like protein is synthesized less in developed follicles and more in its initial stage of development. In buffaloes, FF quantitative profiling of proteins remains similar during monsoon and winter as both are favourable seasons for its breeding. Further studies involving breeding and non breeding season would be more useful to know its seasonal variations. In future, identification and characterization of any season specific protein will be a biomarker to enhance reproductive efficiency of buffaloes during its non-breeding season.

Table 1: % Relative quantity (mean±SEM) of electrophoretic bands in different sized follicles and during monsoon and winter season.

Band (MW)	Season	Follicle size			Pooled *
		Small	Medium	Large	
1 (246.0)	Monsoon	0.49±0.12	0.64±0.08	0.49±0.08	0.54±0.08
	Winter	0.33±0.07	0.55±0.05	0.51±0.11	0.47±0.06
	Pooled**	0.42±0.08	0.60±0.08	0.50±0.06	
2 (226.7)	Monsoon	0.45±0.06	0.76±0.09	0.50±0.10	0.57±0.06 a
	Winter	0.29±0.06	0.54±0.13	0.31±0.07	0.35±0.07 b
	Pooled**	0.35±0.05	0.67±0.07	0.42±0.06	
3 (208.4)	Monsoon	0.75±0.11	1.09±0.11	0.80±0.12	0.88±0.07
	Winter	0.64±0.10	1.01±0.18	0.71±0.17	0.79±0.17
	Pooled**	0.71±0.08	1.06±0.12	0.76±0.09	
4 (164.1)	Monsoon	10.20±0.70	11.94±0.50	11.28±0.92	11.14±0.65
	Winter	10.30±1.08	12.10±1.06	10.75±0.89	11.05±0.67
	Pooled**	10.25±0.58	12.01±0.48	11.07±0.75	
5 (137.2)	Monsoon	1.13±0.12	1.32±0.15	1.24±0.20	1.35±0.14
	Winter	0.95±0.16	1.33±0.27	0.76±0.13	1.22±0.11
	Pooled**	1.33±0.14	1.35±0.09	1.24±0.11	
6 (129.5)	Monsoon	1.13±0.12	1.32±0.15	1.24±0.20	1.24±0.11
	Winter	0.95±0.16	1.03±0.27	0.76±0.13	1.01±0.13
	Pooled**	1.06±0.09	1.32±0.14	1.04±0.14	
7 (120.9)	Monsoon	1.51±0.15	1.24±0.16	1.27±0.13	1.34±0.09 a
	Winter	1.07±0.11	1.07±0.11	0.98±0.13	1.02±0.08 b
	Pooled**	1.35±0.11	1.18±0.11	1.15±0.10	

8 (115.5)	Monsoon	0.96±0.09	1.45±0.13	1.14±0.18	1.19±0.11
	Winter	1.45±0.26	1.95±0.42	1.52±0.25	1.64±0.28
	Pooled**	1.17±0.13	1.66±0.19	1.29±0.15	
9 (101.6)	Monsoon	0.58±0.06	0.65±0.08	0.77±0.09	0.67±0.52
	Winter	0.43±0.08	0.68±0.09	0.48±0.06	0.53±0.04
	Pooled**	0.05±0.05	0.66±0.06	0.66±0.07	
10 (95.9)	Monsoon	0.52±0.06	0.65±0.07	0.55±0.05	0.57±0.05 [a]
	Winter	0.27±0.04	0.43±0.10	0.40±0.09	0.37±0.04 [b]
	Pooled**	0.42±0.05	0.56±0.06	0.49±0.05	
11 (92.7)	Monsoon	0.68±0.08	0.50±0.09	0.57±0.08	0.58±0.06
	Winter	0.81±0.05	0.79±0.09	0.72±0.08	0.78±0.05
	Pooled**	0.73±0.05	0.62±0.07	0.63±0.06	
12 (85.3)	Monsoon	0.12±0.02	0.10±0.01	0.14±0.03	0.12±0.02
	Winter	0.09±0.01	0.11±0.01	0.10±0.02	0.10±0.01
	Pooled**	0.11±0.01	0.10±0.01	0.12±0.02	
13 (81.6)	Monsoon	0.49±0.06	0.38±0.05	0.50±0.07	0.46±0.01
	Winter	0.44±0.06	0.43±0.08	0.38±0.07	0.42±0.05
	Pooled**	0.47±0.04	0.40±0.04	0.45±0.05	
14 (76.9)	Monsoon	1.44±0.27	1.92±0.44	2.06±0.49	1.80±0.34
	Winter	1.44±0.26	1.65±0.24	1.99±0.37	1.69±0.22
	Pooled**	1.43±0.19	1.81±0.28	2.03±0.32	
15 (73.6)	Monsoon	2.87±0.18	2.68±0.29	2.27±0.23	2.61±0.14
	Winter	2.39±0.24	2.91±0.36	2.35±0.23	2.55±0.25
	Pooled**	2.68±0.15	2.77±0.22	2.30±0.16	
16 (69.9)	Monsoon	3.68±0.54	3.23±0.28	3.48±0.44	3.46±0.32
	Winter	4.98±0.90	5.15±1.00	3.90±0.93	4.67±0.78
	Pooled**	4.20±0.49	3.99±0.47	3.64±0.44	
17 (56.1)	Monsoon	49.83±1.21	49.85±0.95	49.91±2.29	49.86±1.00
	Winter	49.20±1.85	44.07±1.74	50.89±2.43	48.05±1.22
	Pooled**	49.58±1.03	47.53±1.09	50.30±1.64	
18 (49.1)	Monsoon	18.45±1.50	17.05±0.81	18.21±1.12	17.90±0.90
	Winter	19.49±0.62	20.11±0.66	18.49±0.99	19.36±0.57
	Pooled**	18.87±0.90	18.27±0.64	18.32±0.76	
19 (42.9)	Monsoon	0.50±0.08	0.39±0.05	0.48±0.06	0.46±0.04
	Winter	0.73±0.17	0.74±0.14	0.69±0.17	0.72±0.14
	Pooled**	0.59±0.08	0.53±0.07	0.57±0.08	
20 (38.8)	Monsoon	0.41±0.07	0.49±0.07	0.52±0.03	0.47±0.04
	Winter	0.45±0.07	0.48±0.08	0.37±0.09	0.43±0.07
	Pooled**	0.43±0.05	0.49±0.05	0.46±0.04	
21 (37.1)	Monsoon	0.83±0.11	0.51±0.06	0.50±0.05	0.61±0.05
	Winter	0.82±0.10	0.66±0.09	0.44±0.06	0.64±0.06
	Pooled**	0.83±0.07 [a]	0.57±0.05 [b]	0.48±0.04 [b]	
22 (33.4)	Monsoon	1.96±0.12	1.23±0.13	1.11±0.16	1.43±0.09
	Winter	1.70±0.34	1.58±0.33	1.62±0.31	1.63±0.30
	Pooled**	1.85±0.15	1.37±0.15	1.31±0.16	
23 (31.1)	Monsoon	0.31±0.05	0.28±0.05	0.36±0.06	0.31±0.03
	Winter	0.31±0.18	0.20±0.05	0.23±0.06	0.25±0.06
	Pooled**	0.31±0.07	0.25±0.04	0.31±0.04	
24 (30.0)	Monsoon	0.37±0.05	0.30±0.03	0.40±0.05	0.36±0.03
	Winter	0.29±0.06	0.29±0.05	0.30±0.07	0.28±0.03
	Pooled**	0.33±0.04	0.29±0.03	0.35±0.04	

Note: Superscripts bearing different small letters within a row or column for a particular band differs significantly(P<0.05).
* Refers to mean value of band irrespective of size of follicle
** Refers to mean value of band irrespective of season

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