PROTEO-BIOCHEMICAL CHARACTERIZATION OF OVARIAN FOLLICULAR CYSTS IN BUFFALOES *(Bubalus bubalis)*

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ABSTRACT

Follicular cyst is one of the major factors affecting the fertility of buffaloes. The present study was conducted to assess the proteo- biochemical characters of the follicular fluid (FF) and granulosa cells (GCs) of normal and cystic follicles of buffaloes. Ovaries were collected from slaughtered buffaloes and follicles were classified based on their diameter as: small (SF: < 4mm), medium (MF: 4 – 9 mm), large (LF: > 9 mm) and cystic (CyF: > 17mm). FF and GC were collected aseptically and subjected for further studies. Biochemical analysis of FF revealed significantly high concentrations of glucose and triglyceride in CyF when compared to LF, which could be due to poor metabolic activities of the damaged follicular cells. Protein fraction analysis of the FF and GC revealed increased expression of proteins in the molecular weight ranges of around 30,70 and 200 kDa relating to insulin like growth factor binding protein, heat shock protein 70 and vasculo endothelial growth factor respectively in CyF. It was concluded that poor utilization of proteins and deranged metabolic and angiogenic proteins in the follicular microenvironment would have led to the anovulatory status and persistence as cyst in buffaloes.

Key words: Buffaloes, Follicular cysts, Biochemical characters, Protein profile

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INTRODUCTION

Fertility of a dairy animal is the backbone of the profitable dairy industry, but it is hindered by various reproductive pathologic conditions. Cystic degeneration of follicle is one such ovarian dysfunction that leads to reproductive failure in cattle and buffaloes (Das and Khan, 2010). With the advent of ultrasonographic demonstrations, an ovarian follicular cyst is generally defined as a follicle

like fluid filled structure, with a diameter of >20 mm and persisting for more than six days on the ovarian surface in the absence of a corpus luteum and clearly interfering with normal ovarian cyclicity (Silvia *et al.,* 2002; Braw-Tal *et al.,* 2009).

Follicular cyst is a consequence of a mature follicle that fails to ovulate at the appointed time during the oestrous cycle. The etiology of cystic ovarian follicles seems to be multifaceted and linked with hormonal, immunological and metabolic imbalances within the follicular microenvironment (Lima *et al.,* 2019), which is composed of follicular fluid (FF) and follicular cells, the granulosa and theca interna. FF originates from exudates of plasma and partly of secretions within the follicle. The locally produced components of FF are related to the metabolic activity of follicular cells (Gerard *et al.,* 2002). The functional status of follicular cells is reflected in the biochemical and protein profile of FF in buffaloes (Joy *et al.,* 2015) based on which, it was hypothesized that the proteo-biochemical components of the follicular microenvironment will be deranged in the cystic follicles (CyF) and exploration of these deviations will aid in deciding the therapeutic or preventive measures to alleviate the ailment. Hence the present research was planned to estimate important biochemical components and to document electrophoretic pattern of proteins in the FF and granulosa cells of CyF, in comparison with normally developing follicles in water buffaloes (*Bubalus bubalis*).

MATERIALS AND METHODS

Ovaries were collected individually from sexually mature buffaloes which were slaughtered in Chennai Corporation abattoir and utilized for the study. The individual pair of ovaries were collected from each animal immediately after slaughter, washed in phosphate buffered saline (PBS). The collected ovaries were transported at 37ºC in PBS to the Centralized Embryo Biotechnology laboratory, Department of Animal Biotechnology, Chennai within 30 minutes after slaughter.

Classification of follicles

Ovaries were washed and length (L) and breadth (B) of the surface antral follicles were measured (in mm) using metric scale. Diameters of the structures were arrived by calculating the average of L and B. The follicles were classified as small (SF: < 4mm), medium (MF: 4 – 9 mm), large (LF: > 9 mm) representing the normal recruited, developing and dominant follicles respectively (Satheshkumar *et al.,* 2016). Follicles were considered cystic (CyF) when the diameter is > 17mm (Silvia *et al.,* 2002).

Collection of follicular fluid and cells

In all the categories, the FF was aspirated using a sterile hypodermic insulin syringe and dispensed in separate petri dishes. The FF from five to ten SFs, four to five MFs, one to two LFs and single CyFs were considered as a single aliquot respectively. The aspirated FF was screened under zoom stereo microscope and oocytes were recovered. After the oocyte recovery, the FF was transferred to 1.5ml micro-centrifuge tube and centrifuged at 10,000 rpm for 10-15 minutes at 4ºC. The supernatant FF and pelleted granulosa cells (GC) from the four categories were stored in

separate micro-centrifuge tubes at -20ºC for further analysis. A total of 10 aliquots were studied in each category.

Biochemical assay

Aliquots of FF samples of SF (SFF), MF (MFF), LF (LFF) and CyF (CyFF) were thawed at room temperature and subjected to biochemical analysis of three major metabolites viz., glucose (Glu), total proteins (TP), and triglycerides (TG). The determination of metabolite levels in FF was done by colorimetric method using Auto Analyzer (A15 Biosystems) by appropriate commercial kits as described by Nichol *et al.* (1992) at Centralized Clinical Laboratory, Madras Veterinary College, Chennai. All the assays were carried out according to the manufacturer's instructions.

Protein profile of follicular fluid and cells

The electrophoretic pattern of proteins in the FF and GCs of MF, LF and CyF were studied. Total protein was extracted from GC pellets of all the three categories as described by Gerard *et al.* (1998) and TP was estimated by Bradford protein assay. Based on the TP concentration the FF and GC samples were evenly corrected to a concentration of 6mg/dl with 1X PBS and further diluted to 1:10 ratio with 1X PBS and the protein profile were studied by standard SDS-PAGE method with a 12% separating gel and a 5% stacking gel. Broad range molecular-weight (MW) standards (Bio-Rad) were also routinely loaded. Electrophoresis was performed at a constant intensity of 50 mA / gel. At the end of migration, gels were stained with Coomassie blue stain overnight at room temperature and destained by repeated rinsing in gel de-staining solution. The enhanced chemiluminescence detection system (Amersham Life Science, Buckinghamshire, UK) was used to detect polypeptides.

Statistical analysis

Data on follicular biochemical parameters of FF were analysed by Student's *t*-test. SPSS.10.0® software was used for analysis of data was carried out as per Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

The study on physical characteristics revealed that FF from SF, MF and LFs were generally straw yellow to yellow in colour and those from CyFs were dark yellow to orange in colour which was in agreement with the earlier observations of Maniwa *et al.* (2005) and Khan *et al.* (2011). The former authors suggested that the darker colouration of CyFF could be due to the interfusion of blood into the CyFs. CyFF was slightly viscous when compared to FFs from normal follicles and frequently coagulated in petri plates, if left unprocessed.

Biochemical factors in follicular fluid

The data on biochemical analysis of Glu, TP and TG in SF, MF, LF and CyF were represented in Table. In general, the Glu concentration increased significantly ($P < 0.01$) as the follicle size increased as observed earlier by Nandi *et al.* (2008) and Kor *et al.* (2013). The later authors further substantiated

that LFs have the ability to filter and reserve the high concentrations of Glu from blood for utilization in their development to the mature Graafian follicle. The Glu concentration in CyF was significantly ($P < 0.01$) higher when compared to normal LFs, similar to the findings of Nath *et al*. (2014) in sows. It could be assumed that relatively smaller number of GCs in CyFs might be consuming Glu from a relatively larger amount of FF, as indicated by Gosden *et al.* (1988), leading to the accumulation of metabolite.

The TG concentration in SFF was significantly ($P < 0.01$) higher than that of MFF and LFF corroborating with the earlier report (Ellah *et al.,* 2010). The TG in FF mainly originates from the local metabolic processes because it could not pass through the follicular membrane as it requires very low-density lipoprotein fraction for transportation which is too large to pass through the barrier (Grummer and Carroll, 1988; Leroy *et al.,* 2004). Based on an *in-vitro* culture study, Kim and Tilly (2001) opined that TG might serve as an alternative energy source for the follicular metabolism. Thus, it could be assumed that the reduction in TG values in normal MF and LFs when compared to SFs indicated the utilization of TG by the intrafollicular components. On the contrary, TG concentration in CyFF was significantly ($P< 0.01$) higher than LFs, which is a definitive indicator of accumulation of TG due to its poor utilization. There was no significant difference in TP concentrations between the follicular categories.

Based on the biochemical study, it could be concluded that poor metabolism and improper utilization of the resources by the follicular cells might be the major reason for high concentrations of Glu and TG in CyFF when compared to LFF. Maniwa *et al.* (2005) opined that the GC layers were exfoliated and the theca interna cells were thinner in CyFs than those of healthy follicles. Hence, poor

Table. Biochemical parameters of follicular fluid in normally developing and cystic follicles of buffaloes

S. No	Follicular category	Biochemical contents in FF		
		Glucose (mg/dl) (Mean ± SE)	Total Protein (g/dl) (Mean ± SE)	Triglyceride (mg/dl) (Mean ± SE)
1	SF	4.09±0.69^{a}	7.23±0.15^{a}	64.40±4.60^{c}
2	MF	8.86±0.68^{a}	6.57±0.09^{a}	35.80±3.20^{a}
3	LF	20.60±0.96^{b}	6.14±0.10^{a}	39.80±5.10^{a}
4	CyF	27.65±5.45^{c}	7.27±0.53^{a}	47.4±4.60^{b}
	Significance	**	NS	**

** Values within columns with different superscripts differ significantly ($P < 0.01$)
NS Not significant ($P > 0.05$)

metabolic profile of CyF could be attributed to the damaged follicular cells which would have caused the functional deficiency leading to anovulatory status.

Protein profile of follicular fluid and follicular cells

The electrophoretic patterns of proteins in the FF and GCs of MF, LF and CyF were depicted in the Fig. SDS-PAGE analysis of FF and GCs revealed that a protein band with a MW just below 78 kilo Daltons (kDa) was intensely expressed in CyF than the other two categories of normal follicles. Heat shock protein 70 (Hsp70), a 70kDa protein, is usually upregulated in response to cell stress. Increased expression of Hsp70 was documented in FF and GCs of bovine CyFs when compared to normal follicles by Maniwa *et al.* (2005) and Velazquez *et al*. (2013). Stress due to negative energy balance and high ambient temperature seems to be some etiologic factors for ovarian cystic degeneration in cattle. Salvetti *et al.* (2009) reported changes in the expression of receptors for steroid hormones in ovarian cells in association with intense Hsp70 immunostaining in the cells of cystic follicles. Based on these facts, the increased expression of a protein around 70kDa observed in our study could be correlated to Hsp70.

An intense band of protein with a MW of about 30 kDa was present in FF and GC of CyF. Satheshkumar *et al.* (2019) demonstrated that similar MW range of protein was found retained in LFs of acyclic animals but disappeared in cyclic animals, which could be considered as an Insulin like growth factor binding protein (IGFBP) as referred by Mazerbourg and Monget (2018). Persistence of IGFBPs would sequester the IGF and prevented the progress in follicular maturation, steroidogenesis and ovulation as suggested by Braw-Tal *et al.* (2009). The findings of the current study support the fact that high activity of IGFBPs which would have reduced the levels of IGF bioavailability might be associated with cyst formation.

The MW of bovine prothrombin and thrombin was reported to be 68.5 and 33.7 kDa respectively (Inada *et al.,* 1970). Protein bands of these MW ranges were found in MFs but they have lost their intensity in LFs indicating the disappearance of these proteins in the final stages of follicular development. Interestingly, such proteins were intensely expressed in FF and GCs of CyF. Active thrombin catalyzes the polymerization which converts soluble fibrinogen into insoluble fibrin during the cascade of blood coagulation events (Chapin and Hajjar, 2015). *In-vivo* ultrasonographic examination of cystic follicles in buffaloes too revealed sparse intra-follicular fibrous strands (unpublished data). Based on our observation, the coagulating properties of CyFF might be attributed to the presence of these coagulating proteins.

Increased intensity of a protein band just below 200 kDa was observed in the GCs of CyF, but not in MF and LF. Vascular endothelial growth factor (VEGF) and its receptors have been reported in the ovaries of many mammalian species and are considered to be important regulators of ovarian angiogenesis (Reynolds and Redmer, 1998). Elevated levels of VEGF, a 180 kDa receptor kinase, might interact with its receptors in the

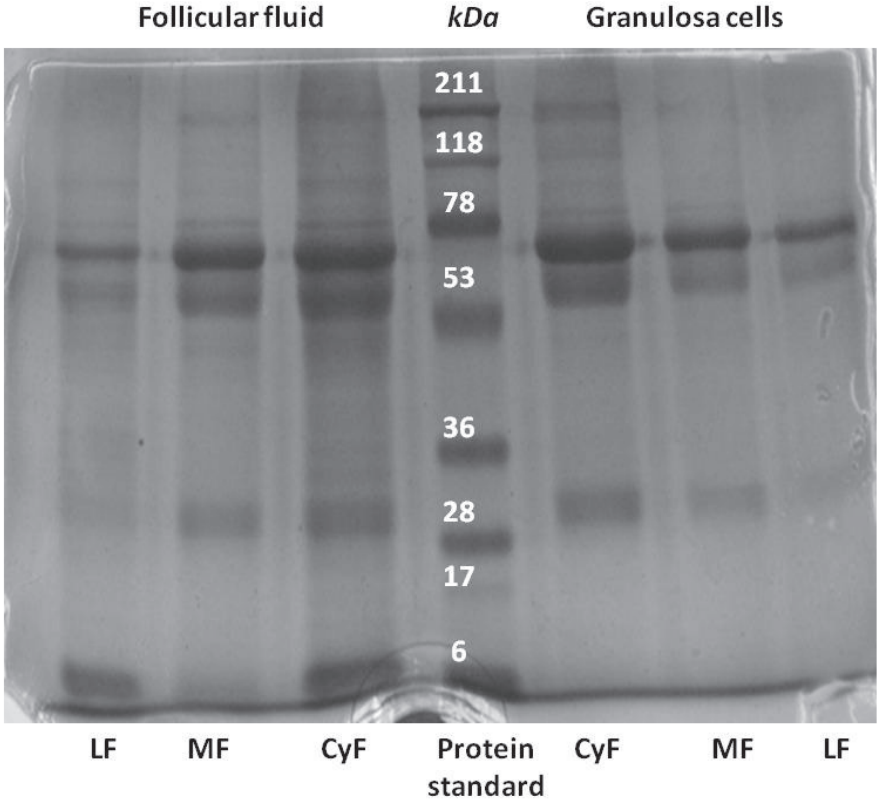

Fig. SDS-PAGE patterns of follicular fluid and granulosa cells in normally developing and cystic follicles of buffaloes

affected ovaries, preventing apoptosis of GC and thus contributing to the continuous growth and persistence of follicles (Greenaway *et al.,* 2004). The implication of VEGF in the etiology of polycystic ovary syndrome was well documented by them in humans. Thus the increased intensity of a protein expression just below 200 kDa in the GCs of CyF could be correlated to VEGF.

We could observe retention and increased expression of certain proteins in the FF and GCs of CyFF relating to patho-physiology of the structure. However, further research with 2D-PAGE and Mass Spectrophotometry will provide ample information about the role of individual proteins in the incidence of cystic ovarian degeneration in buffaloes. Identification of specific proteins might aid in developing protocols for up-regulating / down-regulating them and improve the fertility altogether in buffaloes.

CONCLUSION

High concentrations of Glu and TG in CyFF when compared to normal LF could be correlated to defective metabolism within the follicular microenvironment. Electrophoretic protein profile also revealed retention of major peptides related to stress, failure of the intra-follicular IGF / IGFBP system and angiogenesis. These aberrations in the follicular metabolism would have led to the anovulatory status and persistence as cyst in buffaloes.

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