Protein Profile of Granulosa Cells in Cyclic and Acyclic Buffaloes

S. Satheshkumar*, B. Revathipriya, S. Sakthivel, K. Brindha and M. Parthiban
Department of Animal Biotechnology, Madras Veterinary College
Tamilnadu Veterinary and Animal Sciences University, Chennai – 600 051, Tamilnadu

ABSTRACT

The objective of the study is to analyze the alterations in the protein profile of follicular cells in normal cyclic and acyclic anoestrus water buffaloes. Ovaries from sexually mature buffaloes were collected from abattoir and categorized into two groups viz., i. Cyclic and ii. Acyclic. In both the categories of ovaries, the follicular fluid (FF) was aspirated from all large follicles (≥ 9mm diameter) and small follicles (< 9 mm diameter) separately. FF was centrifuged and four categories of pelleted granulosa cells (CSG and ASG: Granulosa cells of small follicles in cyclic and acyclic groups respectively; CLG and ALG: Granulosa cells of large follicles in cyclic and acyclic groups respectively) were subjected for SDS-PAGE analysis. A total of 30 and 18 bands (from 7.1 to 209.0 kDa) were observed in CSG and CLG categories, while 16 bands in each of the ASG and ALG were recorded. It was observed that 40 per cent of bands present in the CSG group were not observed in CLG group. On the contrary, almost all the proteins found in ASG group were retained in the ALG group. It can be inferred that a group of small molecular weight granulosa cell proteins, correlating to IGF / IGFBP system, play a key role in providing a favorable proteo-genomic environment in the early stages of follicular development. In acyclic animals, retaining of such proteins in the large follicles indicated that the non-availability of metabolic factors needed for final maturation of follicles.

Key words: Buffaloes, Anoestrus, Follicular cells, Protein profile analysis

INTRODUCTION

Water buffaloes (*Bubalus bubalis*) are the major contributors of the dairy sector in the south Asian countries. However the milk production parameters of buffaloes are dependent on their reproductive efficiency. Acyclicity due to anovulatory anoestrus is the major disturbance reported to be affecting the reproductive efficiency of buffaloes (Abraham, 2017). Inspite of being acyclic, the anoestrus cattle are found to exhibit usual ovarian follicular wave activity, but the follicles that are able to attain ovulatory size failed to induce oestrus signs and to ovulate (Ghuman *et al.,* 2010; Satheshkumar *et al.,*2012). Khan *et al.* (2013) reported a lower oestradiol: Progesterone ratio in the follicular fluid (FF)

[1] Present address: Professor and Head, Department of Veterinary Gynaecology and Obstetrics, Veterinary College and Research Institute, TANUVAS, Tirunelveli – 627 358, Tamil Nadu, India
[2] M.Phil. Scholar, Department of ABT, MVC, Chennai - 600 007.
[3] Senior Research Fellow, Department of ABT, MVC, Chennai - 600 007.
[4] Associate Professor, Department of ABT, MVC, Chennai - 600 007.
[5] Professor and Head, Department of ABT, MVC, Chennai – 600 007.
*** Corresponding author Email:** drsatheshkumar6@rediffmail.com

of acyclic buffaloes. Further anovulatory anoestrus is possibly associated with insufficient production of steroid hormones by the growing preovulatory follicle, which is normally characterized by a high steroidogenic synthesis by the cytochrome P450scc and P450arom enzymes (Diaz *et al.,* 2012). These findings were indicative of failure in follicular maturation and defective steroidogenic activity in the follicular micro-environment which affected the oestrus expression and ovulation thereon.

The functional status of follicular cells is reflected in biochemical and protein profile of FF in buffaloes (Joy *et al.,* 2015). Changes in mRNA expression of various genomic factors (gonadotropin receptors, growth factors and their binding proteins etc.,) associated with different stages of follicular growth and atresia has been appreciated in follicular cells (Mishra *et al.,* 2015). Apart from the changes in mRNA expression pattern, it was hypothesized that the proteomic factors of follicular cells will alter in relation to physiological status of follicular development. Hence the present research was conducted to study the protein profile of granulosa cells in cyclic and acyclic buffaloes by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and to analyse the electrophoretic patterns between the two groups of animals.

MATERIALS AND METHODS

Source and preparation of ovaries

Ovaries were collected individually from sexually mature buffaloes (*Bubalus bubalis*) from 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) and transported at 37ºC in PBS to the laboratory within 30 minutes after collection.

The pair of ovaries was categorized into two groups viz., i. Cyclic: If prominent corpus luteum (CL) tissue is present in any one of the ovaries and ii. Acyclic: If no CL is present in both the ovaries.

In both the categories of ovaries, the FF was aspirated from all large follicles ($\geq$ 9mm diameter) and small follicles ($<$ 9 mm diameter) separately using a sterile hypodermic insulin syringe and dispensed in separate petri dishes. The FF from one to two large follicles and four to five small follicles were considered as a single aliquot respectively. The aspirated FF was screened and oocytes were recovered. After the oocyte recovery, the FF was transferred to 1.5ml micro-centrifuge tube and centrifuged at 10,000 g for 10-15 minutes at 4ºC. The supernatant FF was stored in separate micro-centrifuge tubes and four categories of pelleted granulosa cells (CSG and ASG: Granulosa cells of small follicles in cyclic and acyclic groups respectively; CLG and ALG: Granulosa cells of large follicles in cyclic and acyclic groups respectively) were stored at -20ºC for protein analysis. A total of 10 aliquots were studied in each category.

Total protein was extracted from granulosa cell pellets of all the four categories as described by Gerard *et al.* (1998). The total soluble protein (TP) was estimated by Bradford protein assay. Based on the TP concentration the extracted samples were evenly corrected to a concentration of 6mg/

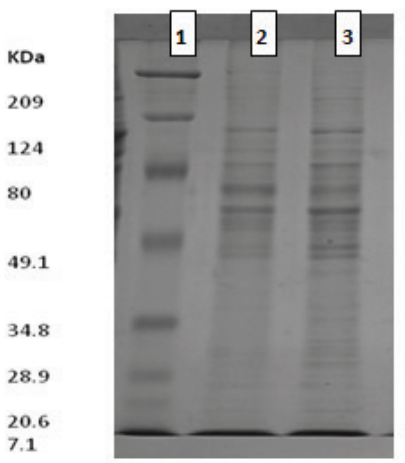
dl with 1X PBS and further diluted to 1:10 ratio with 1X PBS and the protein profile were studied by standard SDS-PAGE method with a 12% separating gel and a 5% stacking gel. Broad range molecular-weight (MW) standards (Bio-Rad) were also routinely loaded. Electrophoresis was performed at a constant 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.

RESULTS AND DISCUSSION

Comparative SDS-PAGE patterns of granulosa cells of small and large follicles in cyclic and acyclic buffaloes are presented in Table 1, Fig. 1 and Fig.2 respectively.

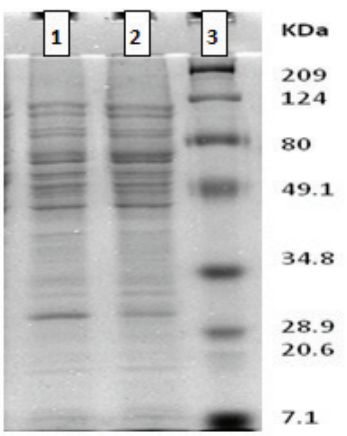
Table – 1. SDS-PAGE patterns of granulosa cells in cyclic and acyclic buffaloes

Molecular weight (kDA)	No. of detectable bands			
	CSG	CLG	ASG	ALG
>124 – 209	4	3	--	--
>80 – 124	4	3	4	4
>49.1 – 80	6	6	4	4
>34.8 – 49.1	6	4	4	5
>28.9 – 34.8	7	1	3	3
>20.6 – 28.9	2	--	--	--
7.1 – 20.6	1	1	1	--
Total no. of bands	**30**	**18**	**16**	**16**



Lane 1 : Protein marker (Broad range)
Lane 2 : Large follicular cells
Lane 3 : Small follicular cells

Fig.1: SDS-PAGE patterns of granulosa cells in cyclic buffaloes



Lane 1 : Large follicular cells
Lane 2 : Small follicular cells
Lane 3 : Protein marker (Broad range)

Fig.2: SDS-PAGE patterns of granulosa cells in acyclic buffaloes

SDS-PAGE analysis of follicular cell proteins revealed differences in the overall electrophoretic pattern between acyclic and cyclic buffaloes. A total of 30 and 18 bands of MW ranging from 7.1 to 209.0 kDa were observed in CSG and CLG categories, while 16 bands in each of the ASG and ALG were recorded. However, Khan *et al.* (2013) reported that there were no qualitative alterations in the protein component of the FF during acyclicity in buffaloes. A maximum of 30 protein bands were detected in the granulosa cell lystaes of cyclic buffaloes in the present study which is in accordance with the findings of Kulkarni (1990) who recorded 26 protein bands in bubaline FF.

In cyclic animals, 40 per cent of protein bands (especially in the range of 20.6 – 34.8 kDa) present in the small follicles obviously disappeared in the large follicles. On the contrary, in acyclic group, all the proteins present in the small follicles were found retained in the large follicles. It can be inferred that a group of small MW granulosa cell proteins play a key role in building up the essential nutrients, hormones and growth factors thus providing a favorable proteo-genomic environment in the early stages of follicular development. Under the gonadotrophin stimulus, these small MW proteins might decrease in concentration and release the sequestered metabolic factors during final stages of follicular development enabling the attainment of dominance. In acyclic animals, retaining of such proteins in the large follicles indicated that the bioavailability of metabolic factors needed for final maturation of follicles was prevented.

A prominent couplet of bands is observed just below the 49.1 kDa protein range in CSG group, which is much faintly expressed in granulosa cells of CLG group. This couplet of bands observed can be correlated to 44–42 kDa native IGFBP-3 as reported by Mazerbourg and Monget (2018). In cyclic animals, the disappearance of IGFBP-3 in the larger follicles signifies the bioavailability of free IGF which ensures follicular maturation. Likewise, an intense band of protein with a MW of about 30 kDa was present in small follicular cells of both the cyclic and acyclic group, which was found to be retained in large follicles of acyclic animals but disappeared in cyclic animals. Thus it could be speculated that the disappearance of that protein might have a major role in terminal follicular development. Similarly, Nandi *et al.* (2006) demonstrated a 30.1 kDa ovine intrafollicular factor that was inhibitory for both cumulus and granulosa cell proliferation *in vitro*. Bridges *et al.* (2002) and Mazerbourg and Monget (2018) stated that the apparent MW of IGFBP-5 ranged between 29 and 30 kDa. The findings confirmed the fact that the concentrations of IGFBPs decrease during final stages of follicular growth, leading to an increase in IGF bioavailability for maturation, oestradiol production and ovulation in normally cycling animals (Fortune *et al.,* 2004). On the other hand, IGFBPs persist in the follicular cells thus sequestering the IGF and preventing the progress in follicular maturation (Braw-Tal *et al.,* 2009). Thus the failure in function of the IGF system in granulosa cells might contribute for the deficient follicular metabolism and steroidogenesis leading to anovulatory anoestrus condition.

No high MW proteins in the range between 124 to 209 kDa were detected in both the follicular categories of acyclic group. On the contrary, 3-4 bands were observed in this range in cyclic animals. In accordance with our findings, Gerard *et al.* (1998) demonstrated presence of a 200-kDa protein in granulosa cells lysates recovered from equine preovulatory follicles. They attributed that this stage specific expression of high MW protein might be involved in the differentiation and maturation mechanisms occurring in the follicle during the preovulatory period. Rivera and Fortune (2003) described Pregnancy-associated plasma protein A (PAPP-A), which is composed of two 200-kDa disulfide-bonded subunits, is the major protease that lyses the IGFBP-4/-5 and has been detected in the FF of bovine preovulatory follicles (Mazerbourg *et al.,* 2001). In light of the present results, it is tempting to speculate that such high MW proteolytic system is active in the cyclic animals, but not in the acyclic animals.

Eventhough we have not conducted specific protein characterization studies, based on the comparisons with previous literatures it could be concluded that low MW proteins in the granulosa cells, correlating to IGF / IGFBP system, play a key role in the follicular maturation. Factors affecting the final stages of development of dominant follicle are closely related to the metabolic status of animals. Reduced plasma concentrations of metabolic hormones (insulin) and related growth factors (IGF) were reported in nutrient-restricted anoestrus cows and these aberrations are thought to have direct effects on follicular dysfunction (Spicer and Echternkamp, 1995). Thus the study confirmed the hypothesis that the proteomic factors of follicular cells were altered in relation to the physiological status of follicular development.

It could be concluded that failure of the intra-follicular IGF / IGFBP system, probably due to deficient nutritional status, lead to ovulatory disturbances and follicular dysfunction in acyclic buffaloes.

REFERENCES

Abraham, F. (2017). An overview on functional causes of infertility in cows. Journal of Fertilization: In Vitro - IVF-Worldwide, Reproductive Medicine, Genetics & Stem Cell Biology, **5**:203

Braw-Tal, R., Pen, S and Roth, Z. (2009). Ovarian cysts in high-yielding dairy cows. *Theriogenology*, **72**:690–698.

Bridges, T.S., Davidson, T.R., Chamberlain, C.S., Geiser, R.D and Spicer, L.J. (2002). Changes in follicular fluid steroids, insulin-like growth factors (IGF) and IGF-binding protein concentration, and proteolytic activity during equine follicular development. *Journal of Animal Science*, **80**179-190.

Diaz, D., Galina, C.S., Fiordelisio, T., Rubio, I., Alarcon, M.A., Rodriguez. A.D and Orihuela, A. (2012). Relationship between growth of the preovulatory follicle and its steroidogenic activity on the onset and expression of estrus behavior in CIDR-treated Bos indicus cows: An observational study. *Physiology & Behavior*, **107**:262–270.

Fortune, J.E., Rivera, G.M and Yang, M.Y. (2004). Follicular development: the

role of the follicular microenvironment in selection of the dominant follicle. *Animal Reproduction Science*, **82–83**:109–126.

Gerard, N., Duchamp, G., Goudet, G., Bezard, J., Magistrini, M and Palmer, E. (1998). A High-Molecular-Weight Preovulatory Stage-Related Protein in Equine Follicular Fluid and Granulosa Cells. *Biology of Reproduction*, **58**:551-557

Ghuman, S.P.S., Singh, J., Honparkhe, M., Dadarwal, D., Dhaliwal, G.S and Singh, S.T. (2010). Fate of dominant follicle in summer anestrus buffaloes. *Indian Journal of Animal Reproduction*, **31:**7-10

Joy, S., Deshmukh, B.T., Dhaware, S.D and Borkar, S.D. (2015). Follicular fluid protein profile in buffalo. *Veterinary Science Research Journal*, **6:**71-79.

Khan, F.A., Das, G.K., Pande, M., Singh, R and Ghosh, S.K. (2013). Evaluation of follicular atresia and electrophoretic pattern of follicular fluid proteins in acyclic buffalo (*Bubalus bubalis*). *Buffalo Bulletin*, **32:**265-269.

Kulkarni, B.A. (1990). Follicular fluid proteins of the India buffalo. Proceedings of II World Buffalo Congress held in India during 12-16 December 1988 (Volume III). *Physiology and Reproduction*. pp. 233-238.

Mazerbourg, S and Monget, P. (2018). Insulin like growth factor binding proteins and IGFBP proteases:A dynamic system regulating the ovarian folliculogenesis. *Frontiers in endocrinology*, **9:**1-10.

Mazerbourg, S., Overgaard, M.T., Oxvig, C., Christiansen, M., Conover, C.A., Laurendeau, I., Vidaud, M., Tosser-Klopp, G., Zapf, J and Monget, P. (2001) Pregnancy associated plasma protein-A (PAPP-A) in ovine, bovine, porcine, and equine ovarian follicles: involvement in IGF binding protein-4 proteolytic degradation and mRNA expression during follicular development. *Endocrinology*, **142**:5243–5253.

Mishra, S.R., Bharati, J., Bharti, M.K., Singh, G and Sarkar, M. (2015). Expression and localization of Fibroblast Growth Factor 10 (FGF10) in ovarian follicle during different stages of development in buffalo. *Asian Journal of Animal and Veterinary Advances*, **10**:433-442.

Nandi, S., Kumar, V.G., Gupta, P.S.P., Ramesh, H and Manjunatha, B.M. (2006). Effect of ovine follicular fluid peptide on follicle, oocyte, and somatic cell culture in buffalo (*Bubalus bubalis*). *Animal Reproduction*, **3**: 61-69.

Rivera, G.M and Fortune, J.E. (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

Satheshkumar, S., Brindha, K., Devanathan, T.G., Kathiresan, D., Kumanan, K and Asokan, S.A. (2013) Ultrasonographic

characterization of follicular turnover in postpartum anoestrus crossbred cattle. Compendium on 'National Symposium and XXVIII annual Convention of ISSAR, Khanapara, Guwahati, Assam, November 21-23, 2012; pp: 234.

Spicer, L.J and Echternkamp, S.E. (1995). The ovarian insulin and insulin-like growth factor system with an emphasis on domestic animal. *Domestic Animal Endocrinology*, **12**:223–245.