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Expression profile of follicular genes vis-à-vis season and cyclicity in buffalo

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Folliculogenesis is controlled by factors and hormones that play critical role during follicular recruitment, selection and dominance. Studies have reported the role of many expressed genes in dominant follicle of cattle (Ken-go Hayashi *et al.* 2010). Studies showed that dominant follicle (DF) showed up-regulation of genes controlling estradiol (E2) synthesis, anti-apoptosis, cell proliferation and gene transcription (Evans *et al.* 2004). Studies in buffaloes on follicular genes have reported activation of various biological pathways inducing several growth factors and peptides especially genes associated with steroidogenesis in pre-ovulatory follicle development (Jyotsna *et al.* 2009, Rao *et al.* 2011). But such studies failed to deduce the seasonal effect of follicular gene expression pattern in buffaloes and still remains to be elucidated. In the light of the above context, the present study was designed to deduce the expression profile of follicular genes with respect to seasons and cyclicity in buffaloes.

The study was conducted on Murrah buffaloes maintained at the Institute. The study was conducted during summer (May to August) and winter (October to February). Post-partum multiparous animals (21) aged between 4.5 and 6.5 years and body weight ranging between 400 and 550 kg (BCS>3) were divided in 3 groups with 7 acyclic and 7 cyclic during summer and 7 cyclic buffaloes during winter. All animals were managed on semi-intensive system under uniform feeding and breeding management.

All the experimental procedures were carried with the approval of the Institutional Animal Ethical Committee (IAEC). Repeated alternate day transrectal ultrasonographical examinations of ovarian activity was made in all buffaloes by a single operator using a B mode ultrasound scanner equipped with an intraoperative 7.0 MHz microconvex transducer. *In situ* follicular sampling in follicles (acyclic: follicle >10mm diameter; cyclic: pre-ovulatory follicle >10mm diameter)) sampling was done during winter and summer following standard procedures. Follicular fluid sampling was carried out as per Pieterse *et al*. (1988). Collected follicular fluid was centrifuged at 1,500 rpm for 10 min and the pellet obtained was washed with normal saline and then with nuclease free water. It was subsequently used for RNA isolation with kit. This was followed by cDNA synthesis using kit. The total RNA extracted was quantified and purity of extracted RNA was deduced from the ratio of absorbance at 260 nm and 280 nm (A260/280) which ranged between 1.6 and 1.8. For cDNA synthesis 2 µg of RNA from each sample whose genes' expression has to be studied was used. Primers used in the study for gene expression are shown in Table 1.

Table 1. Primers, annealing temperature and product size for the genes under study

Gene/accession number	Primers (5´-3´)	Annealing temperature (°C)	Product size (bp)
GAPDH/U85042	F: CAAGGTCATCCATGACCA R: AAGGCCATGCCAGTGA	56	215
BCL2/U92434	F: CTTCGCCGAGATGTCCA R: GGTTCAGGTACTCGGTCA	58.5	215
Aromatase/U18447	F: TTGTCAGCTAGGCAGGA R: AGCCTAGTAGGCTGCAA	56.5	215
Caspase 3/ AY575000	F: CTGTCGATCTGGTACAGA R: GGGTCCGTTGGTTCCA	57	214
LHr/ U20504	F: CTCCCCATGGATGTGGA R: GAGATTGGTGCCATGCA	57	212
HSP70/HM025989	F: GATCGACTCCCTGTTCGA R: TGAAGAAGTCCTGCAGCA	56	210

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Real-time PCR was performed with a kit following manufactures' protocol. cDNA template (2 µL) for each known simple was added to 0.2 ml PCR strips in triplicates along with no template controls containing-qPCR master mix (10µL), 0.5 µl (10 pmol) of forward and reverse primer. The PCR conditions: Initial activation 95°C for 15 min (1 cycle) followed by 40 cycles of denaturation 95°C for 15 sec, gene specific annealing temperaturs of 56–58.5°C for 30 sec followed by extension at 72°C for 1.30 min and for melt curve analysis: 1 cycle each of 95°C for 15 sec, 60°C for 1 min, 95°C for 30 sec and gradual increment from 60°C to 95°C @ 0.5°C/sec and continuous fluorescence measurement. Data were acquired during the annealing step and the specific products obtained for each gene were purified and electrophoresis was done for confirmation. Melt curve analysis was performed for each gene to confirm the presence of a single peak. Q-RT-PCR data (Ct values) was analyzed using the comparative Ct method. Efficiency-corrected relative quantification of mRNA was obtained by Pfaffl (2001).The obtained data were analyzed statistically as per standard procedure using SAS software (Snedecor and Cochran 1989). Gene expression data were analyzed with ANOVA and individual differences were determined by Student's t-test. All results were regarded significant at $P<0.05$.

The hypothesis of the present study was to deduce the changes in genes expression profile arising due to seasons and cyclicity. Ultrasonographical examinations of buffaloes ovarian follicular dynamics on alternate days resulted in identification of dominant follicle (>10 mm) and pre-ovulatory follicles in acyclic and cyclic buffaloes, respectively across seasons. Follicular sampling by ovum pick-up technique retrieved 0.50, 0.70 and 0.90 ml of follicular fluid in acyclic, summer cyclic and winter cyclic buffaloes, respectively. Moreover RNA isolated from follicular cells yielded RNA of concentration of 400 ng/µl with A280/260 ratio of 1.8–1.9. The synthesis of cDNA was checked with housekeeping gene (GAPDH) and the gene specific product of 210–215 bp at specific annealing temperature (Table 1). In the present study expression profile of key genes, viz. aromatase, LHr, caspase 3, BCL2 and HSP70 was studied in folicular cells in cyclic buffaloes during different seasons and cyclicity. Efficiency-corrected relative quantification of mRNA was obtained by Pfaffl (2001). Efficiencies of primer were determined by serial dilution of template cDNA sample and running in triplicate. The efficiencies for BCL2, aromatase, caspase 3, LHr and HSP70 were 95, 98, 94, 88 and 89%, respectively. Accordingly mRNA expression levels of genes, viz. aromatase, LHr, caspase3 and BCL2 were similar between acyclic, summer and winter cyclic groups. In contrast, the relative abundance of HSP70 gene transcripts was significantly higher (1.6 fold) during summer as compared to winter. The expression levels of aromatase, LHr, caspase 3, BCL2 between acyclic and cyclic summer and winter were nonsignificant ($P>0.05$) depicting similar expression of genes during folliculogenesis irrespective of cyclicity and during season. Though it may be speculated that the production of estradiol by aromatase might decrease in the follicles during summer and acyclicity (Baoand Garverick 1998, Irving-Rodgers *et al.* 2009), but this study did not support the hypothesis. This discrepancy might be due to the species difference as well as the extent and duration of heat stress to which the animals were exposed. In addition nonsignificant difference in aromatase, LHr might reflect the progressive growth nature of the follicles which was also evident from the follicular estradiol and progesterone levels showing positive ratio (unpublished data). This is in agreement with Baoand Garverick (1998) who reported no change in gene expression pattern in pre-ovulatory follicles especially aromatase and LHr gene transcripts. Earlier studies in cattle revealed that any follicle attaining more than 10 mm has the intrinsic capacity to acquire LHr along with concurrent increase in estradiol and LH concentration (Ginther *et al.* 1996). Similarly the follicular cells expressed aromatase and LHr gene transcripts in all 3 groups and there was low or no expression of apoptotic genes depicting less direct effect of heat stress on follicle growth as reported earlier (Hansen 2009). Although heat stress suppresses estradiol production in the dominant follicle (Badinga *et al.* 1993) it is unknown whether this suppression results in granulosa cell apoptosis as in this study where there was nonsignificant expression of apoptotic genes. Interestingly in the present study there was nonsignificant low expression of capsase-3. This is supported by other studies which showed the possibility of apoptosis in the pre-maturation changes in dominant follicle and these pre-maturation like changes are always accompanied with apoptotic follicular cells even in healthy follicles with low percentage (Ginther *et al.*1996) with the presence of apoptosis rate of 5 to 30% in the granulosa cell layer and slight increase caspase-3 even in mature pre-ovulatory follicle (Janowskia *et al.* 2012). In the present study nonsignificant expression of BCL2 in acyclic as well as cyclic animals during summer and winter was similar as reported by Evans *et al.* (2004). Further BCL2 expression should always be related to BAX expression as higher BAX expression will initiate apoptosis (Valdez *et al*. 2005). On the other hand, expression of BCL2 in present study suggested that the increase might be to counteract the apoptosis in follicles. Furthermore, studies showed that the ratio of BAX and BCL2 is more important than BCL2 alone in determining apoptosis (Valdez *et al.* 2005). On the contrary in the present study, expression of HSP70 was higher i.e. 1.6 fold increase in acyclic and cyclic summer group as compared to cyclic winter group. This difference can be speculated arising due to its protective function of heat shock proteins in follicular environment under heat stress (Badinga *et al.*1993).This is in agreement with the reports of Hansen (2009) and Hansen (2013). Higher expression of HSP70 transcripts can be attributed to heat stress response during summer as per Hansen (2009). This could be due to the protective effect of HSP70 on cumulus cells and oocyte under heat stress. Similar studies in goats showed increased expression of HSP70 transcripts

in PMN cells during summer (Dangi *et al.* 2012). These findings proved that one of the most important responses to heat stress is the acquisition of increased tolerance of the cell to more severe stress conditions by the up-regulation of heat shock proteins. This is facilitated by the heat shock proteins, which restore protein homeostasis to the pre-stress balance by refolding and repairing damaged proteins and stabilizing ribosomal RNA which could be the valid reason for increase of HSP70 gene transcripts during summer. In conclusion, from the present study it can be deduced that the follicular genes, viz. BCL2, aromatase, caspase 3 and LHr showed no significant difference with exception of HSP70 transcripts across seasons and cyclicity in buffaloes.

SUMMARY

Acyclic (7) and cyclic buffaloes (7 in summer; 7 in winter) were used in the present study for studying follicular gene expression profile. For the study, follicular dynamics monitored using ultrasonography was done to screen the follicles > 10 mm both in acyclic buffaloes and pre-ovulatory follicle in cyclic buffaloes. These follicles were used for follicular cells sampling and RNA was isolated from the follicular cells and cDNA synthesis was done. Annealing temperature of the genes understudy, viz. BCL2, aromatase, caspase 3, LHr, HSP70 were 58.5, 56.5, 57, 57 and 56°C, respectively. The specific PCR products for BCL2, aromatase, caspase 3, LHr and HSP70 genes were 215, 215, 214, 212 and 210 bp, respectively. Relative quantification of the candidate genes across seasons and cyclicity revealed no significant difference for BCL2, aromatase, caspase 3 and LHr genes across season and cyclicity, but HSP70 was significantly higher both in acyclic and summer cyclic buffaloes in comparison to winter. In conclusion, follicular genes under the present study, except HSP70, showed no significant difference across seasons and cyclicity in buffaloes.

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REFERENCES

Argov N, Moallem U and Sklan D. 2005. Summer heat stress alters the mRNA expression of selective-uptake and endocytotic receptors in bovine ovarian cells. *Theriogenology* **64**: 1475–89.

Badinga L, Thatcher W W, Diaz T, Drost M and Wolfenson D. 1993. Effect of environmental heat-stress on follicular development and steroidogenesis in lactating Holstein cows. *Theriogenology* **39** (4): 797–810.

Bao B and Garverick H A. 1998. Expression of steroidogenic enzyme and gonadotropin receptor genes in bovine follicles during ovarian follicular waves: a review. *Journal of Animal Science* **76**: 1903–21.

Dangi S S, Gupta M, Maurya D, Yadav V P, Panda R P, Singh G, Mohan N H, Bhure S K, Das B C, Bag S, Mahapatra R, Sharma G T and Sarkar M. 2012. Expression profile of HSP genes during different seasons in goats *(Capra hircus). Tropical Animal Health and Production* **44**: 1905–12.

Evans A C, Ireland J L H, Winn M E, Lonergan P, Smith G W, Coussens P M and Ireland J J. 2004. Identification of genes involved in apoptosis and dominant follicle development during follicular waves in cattle. *Biology of Reproduction* **70**: 1475–84.

Furtado C L M, Priscilavendramini Silva, Marta Fonseca Martinsguimarães, Nicola Vergara Lopes Serão, José Domingosguimarães, Simone Eliza Facioni Guimarães. 2010. Differential expression of genes in follicular cells of swines. *Revista Brasileira de Zootecnia* **39** (5): 1023–28.

Ginther O J, Wiltbank M C, Fricke P M, Gibbons J R and Kot K. 1996. Selection of the dominant follicle in cattle. *Biology of Reproduction* **55**: 1187–94.

Hansen P J. 2013. Antecedents of mammalian fertility: Lessons from the heat-stressed cow regarding the importance of oocyte competence for fertilization and embryonic development. *Animal Frontiers* **3** (4): 34–38.

Hansen P J. 2009. Effects of heat stress on mammalian reproduction. *Philosophical Transactions of the Royal Society B* **364** (1534): 3341–50.

Irving-Rodgers H F, Harland M L, Sullivan T R and Rodgers R J. 2009. Studies of granulosa cell maturation in dominant and subordinate bovine follicles: novel extracellular matrix focimatrix is co-ordinately regulated with cholesterolside-chain cleavage CYP11A1. *Reproduction* **137**: 825–34.

Janowskia, D, Salilew-Wondimb D, Tornera H, Tesfaye D, Ghanemc B N, Tomeka W, El-Sayedc A, Schellander B K and Hölkerb M. 2012. Incidence of apoptosis and transcript abundance in bovine follicular cells is associated with the quality of the enclosed oocyte. *Theriogenology* **78**: 656–69.

Jyotsna U R and Medhamurthy R. 2009. Standardization and validation of an induced ovulation model system in buffalo cows: Characterization of gene expression changes in the periovulatory follicle. *Animal Reproduction Science* **113**: 71–81.

Ken-Go Hayashi, Ushizawa K, Misahosoe and Takahashi T. 2010. Differential genome-wide gene expression profiling of bovine largest and second-largest follicles: identification of genes associated with growth of dominant follicles. *Reproductive Biology and Endocrinology* **8**:11.

Pfaffl M W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29** (9): e45.

Pieterse M C, Kappen K A, Kruip T A and Taverne M A.1988. Aspiration of bovine oocytes during transvaginal ultrasound scanning of the ovaries. *Theriogenology* **30**:751–62.

Rao J U, Shah K B, Puttaiah J and Rudraiah M. 2011. Gene expression profiling of preovulatory follicle in the buffalo cow: Effects of increased IGF-I concentration on periovulatory events. *PLoS ONE* **6** (6): e20754. doi:10.1371/journal.pone.0020754.

Snedecor G W and Cochran W G. 1989. *Statistical Methods*. 8th edn. Iowa State University Press.

Valdez K E, Peder Cuneo S and Turzillo A M. 2005. Regulation of apoptosis in the atresia of dominant bovine follicles of the first follicular wave following ovulation. *Reproduction* **130**: 71–81.