International Journal of Animal Biotechnology
ISSN 2277 – 4122

Available at http://inpressco.com/category/ijab

Original Research Article

Temporal changes in mRNA expression of heat shock protein genes in mammary epithelial cells of riverine buffalo in response to heat stress *in vitro*

Neha Kapila[ÀĊ], Amit Kishore[À], Monika Sodhi[À], Ankita Sharma[À], AK Mohanty[Ḃ], Pawan Kumar[Ċ] and M Mukesh[À*]

[A]National Bureau of Animal Genetic Resources, Karnal, Haryana, India
[B]National Dairy research Institute, Karnal, Haryana, India
[C]Singhania University, Rajasthan, India

Accepted 02 August 2013, Available online October 2013, **Vol.3 (2013)**

Abstract

Exposure to high ambient temperature affects buffalo productivity significantly in hot climate areas. Till date, no data is available on the heat stress response of mammary epithelial cells (MECs) in buffaloes. The present work aimed to examine the long term temporal changes in mRNA expression pattern of various heat shock proteins (HSP) genes in buffalo MECs during heat stress condition. For the study, we utilized the primary mammary epithelial cells of riverine buffalo and were exposed to thermal stress condition at 42^0C for one hour. The cells were subsequently allowed to recover at 37^0C and harvested at different time intervals (30 min to 48 hr) along with control samples (un-stressed). All the HSP (HSP40, HSP60, HSP70, HSP90, and HSBP1) showed immediate induction in their expression after heat shock and remained upregulated at the later stages as well. Amongst these, HSP70 gene showed maximal induction in its expression while HSP40 was found to be second most abundantly expressed HSP. Our present data thus provides the strong clue about the responsiveness of buffalo MECs to heat stress suggesting its suitability as an in vitro model to understand the modulation of buffalo mammary gland expression signature in response to environmental heat load.

Keywords*: Buffalo, cell culture, mammary epithelial cells, heat stress, heat shock protein.*

1. Introduction

Heat stress phenomenon has become a major issue in the era of climate change. It is well documented that stress induced by hot environments lowers productive efficiency in dairy animals (Johnson, 1965; McDowell, 1966; Bond, 1969; Thatcher, 1974; Fuquay et al.,1981; Marai and Habeeb, 2010). Impact of heat stress needs to be minimized so as to maintain animal health status and performance. Several managemental strategies have helped to lower down the stress in dairy animals but to a limited extent. The riverine buffalo has a special significance in the dairy industry in India and contributes more than 50% of total milk production besides its role as draught power and meat source. However, due to dark coat color and sparse hair on skin surface that absorbs solar radiations; riverine buffaloes have less physiological adaptation and exhibit signs of high distress as compared to cattle, when exposed to direct heat stress. The reduction in milk yield in buffaloes during heat stress is a major concern for dairy sector in India. The loss in milk yield during hyperthermia is believed to be due to its negative effects on secretary activity of mammary gland cells. Mammary epithelial cells (MECs) are representative cells of mammary gland and highly sensitive to environmental changes. However, Isolation of epithelial cells from udder tissue of live animal is technically difficult and the animal has to be either sacrificed or the tissue has to be recovered by tissue biopsy. Further, tissue biopsy procedure renders the animal unproductive due to formation of scar leading to mammary gland fibrosis. To overcome these difficulties, MECs under cell culture conditions could be utilized as a suitable *in-vitro* model for evaluating hyperthermia induced damage.

Upon encountering physiological stress, cells produce heat shock proteins (HSPs) that are major proteins induced during cellular responses to various stresses such as heat shock and oxidative stress (Lindquist and Craig, 1998; Multhoff, 2007). These proteins act as molecular chaperons in regulating cellular homeostasis and folding-unfolding of damaged proteins during thermal or any other physiological stresses (Rane et al., 2003; Li and Srivastava, 2004). The *HSP* genes known to be highly conserved, are well characterized in wide range of organisms. Based on molecular weight, these proteins have been classified into two major families; High molecular weight heat shock proteins such as *HSP120*, *HSP90*, *HSP70*, *HSP60*, *HSP*40 and small molecular weight heat shock proteins (s*HSP*27, *HSP*26, *HSP*16,

*Corresponding Author' Tel: +91-1842267918; fax: +911842267654

*HSP*17). The activated expression of molecular chaperons after heat shock, inhibit apoptosis in a variety of cell types (DeMeester et al., 1997; Garcia-Bermejo et al., 1997; Mosser et al., 1997). The comparative analysis for expression of candidate genes associated with hyperthermia in relevant cells could provide an insight to unravel the thermoregulatory mechanism operative in mammary gland. Till date, no information is available for transcriptional response of buffalo MECs towards heat stress. The current study was therefore planned to assess the temporal expression pattern of some of the major HSP genes in buffalo MECs in response to heat stress *in-vitro*.

2. Materials and Methods

2.1 Establishment of Buffalo MECs primary culture

Mammary tissue was collected from slaughter house under aseptic condition and promptly transported to laboratory at 4°C. Tissue samples of $1mm^3$ cubes were transferred onto collagen coated cell culture dishes (Corning, USA) containing DMEM: F12 (Hyclone, Ultah) supplemented with 10 % FBS (PAA, USA) and incubated at 37^0C, 5 % CO_2. For establishment of primary cell culture of MECs, DMEM: F12 growth media was supplemented with Antibiotic-Antimycotic solution (1X), 5µl/mL insulin, 50µM hydrocortisone, 1µg/ml β-estradiol, 5µg/mL holotransferrin, 1µg/mL Progesterone (Sigma, USA). Subsequent passages and selective trypsinization with 0.25% trypsin-0.02% EDTA (Sigma, USA) was made till homogenous MECs were obtained.

2.2 Heat treatment to buffalo MECs

The cells after 10th passages were exposed to heat stress condition at 42^0C for one hour and subsequently allowed to recover at 37^0C and harvested by trypsinization at different time points (30m, 2h, 4h, 8h, 12h, 16h, 24h and 48h) along with control samples (un-stressed). The culture plate marked as control was kept at 37°C throughout the course of the study. The cells were trizolated using ice-cold Trizol (Invitrogen, Carlsbad, California) and were processed for extraction of total RNA.

2.3 RNA purification and cDNA synthesis

Total RNA extracted was subjected to column purification and RNAse free DNase digestion using RNeasy Mini Kit (Qiagen, Germany). RNA concentration and purity was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and stored at -80^0C till further usage. cDNA synthesis was performed using 100 ng RNA, 1 µl dT_{12-18} (Invitrogen Corp. CA), 1 µL 10 mmol/L dNTP mix (Invitrogen Corp., CA), 1 µl random primers (Invitrogen Corp., CA), and 10 µL DNase/RNase free water. The mixture was incubated at 65 °C for 5 min and kept on ice for 3 min. A total of 6 µl of master mix consisting of 4.5 µl 5X First-Strand Buffer, 1 µl 0.1 M DTT, 0.25 µl (50 U) of SuperScript™ III RT (Invitrogen Corp., CA), and 0.25 µl of RNase Inhibitor (10 U) (Promega, WI) was added. The reaction was performed in an Eppendorf gradient cycler using the program: 25 °C for 5 min, 50 °C for 60 min and 70 °C for 15 min. cDNA was then diluted 1:4 (v:v) with DNase/RNase free water.

2.4 Primer Designing and validation

The primers were either designed using Primer express 3.0 software (Applied Biosystem) or selected from literature with minimum amplicon size ranging between 50-115 bp and limited 3' G+C. Primer details for all the heat shock protein genes are given in Table 1. The primers specificity was confirmed in a 20 µL PCR reaction using the same protocol described for qPCR except for the final dissociation protocol. A 5 µl of PCR product was run in a 2% agarose gel stained with ethidium bromide. The accuracy of primer pairs was also ensured by the presence of a unique peak during the dissociation step at the end of qPCR.

2.5 Real-time quantitative PCR (qPCR)

qPCR was performed using LightCycler 480 instrument (Roche, Germany) using 4 µL diluted cDNA with 6 µL of a mixture composed of 5 µL 2 X LightCycler 480 SYBR Green I master mix (Roche, Germany), 0.4 µL each of 10 µM forward and reverse primers, and 0.2 µL DNase/RNase free water. For each gene, samples were run in duplicate along with 6 point relative standard curve with 5 fold dilution plus the non-template control. The reactions were performed with amplification conditions: 2 min at 50°C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. A dissociation protocol with an incremental temperature of 95 °C for 15 s plus 65 °C for 15 s was used to investigate the specificity of the qPCR reaction and the presence of primer dimers. The qPCR expression data for each target gene was extracted in the form of crossing points (Cp) values. The data was acquired using the '*second derivative maximum*' method as computed by the LightCycler Software 3.5 (Roche Diagnostics) and subjected for subsequent analysis.

2.6 Normalization and data analysis

For determination of optimal expression level, the expression data of *HSP* genes were normalized using geometric mean of three most stable housekeeping genes (HKGs) viz., *RPL4*, *EEF1A1* and *RPS23* as determined by geneNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and Bestkeeper (Pfaffl et al., 2004). These three HKGs were identified as most stable genes in one of our previous studies (Kapila et al., 2013). The gene expression data has been normalized using relative quantification $\mathbf{2^{-\Delta\Delta CT}}$ method (Livak and Schmittgen, 2001) and the expression fold changes estimated as up-regulated or down-regulated against control. For normalization of target genes used, the geometric mean of Ct values of best HKGs has been calculated as shown below: $\Delta Ct = C_t$ (Target)-C_t (Endogenous) $\Delta Ct = \Delta C_t$ (Test sample)-ΔC_t (Calibrator sample)

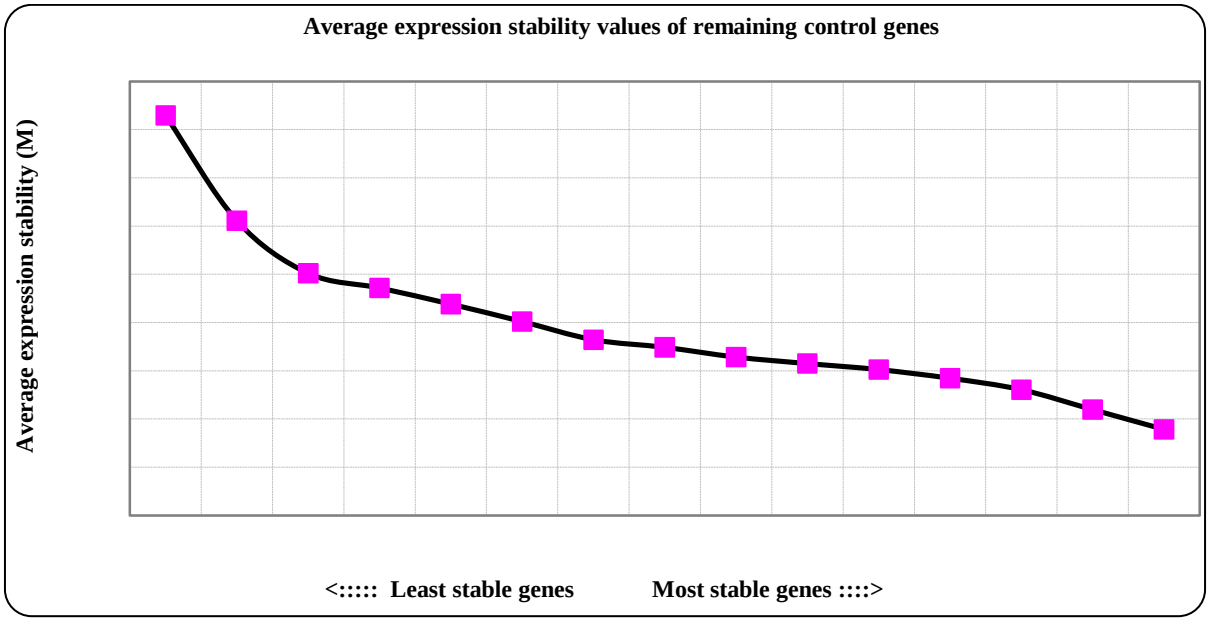

Fig 1: Average expression stability measures (M) for 16 housekeeping genes (HKGs) in buffalo MECs during heat stress. *RPS23*, *RPL4* and *EEF1A1* were observed to be most stably expressed genes.

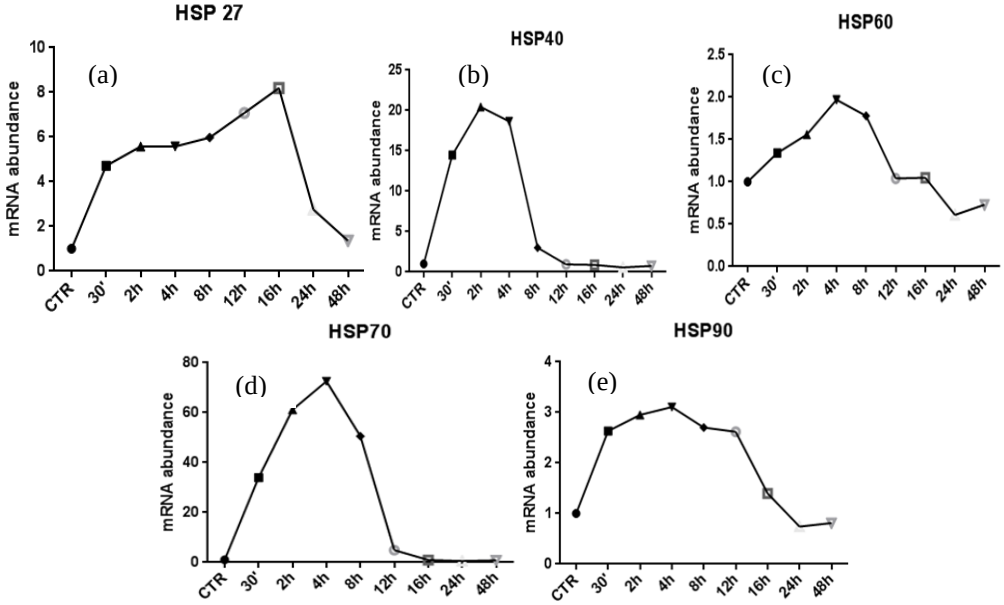

Fig 2: Expression profile of HSP genes (a) *HSP27*, (b) *HSP40*, (c) *HSP60*, (d) *HSP70* and (e) *HSP90* in buffalo MECs in response to heat stress.

3. Results and Discussion

The cell culture of mammary gland specifically in bovine has been explored by several workers to understand the functioning of mammary gland (Huynh et al., 1991; Zavizion et al., 1995; Du et al., 2007; Juan et al., 2008; Li et al., 2009; Hu et al., 2009; Zhao et al., 2010; Anand et al., 2012). The present work utilizes the MECs as *in vitro* model to examine the long term temporal changes in mRNA expression pattern of various heat shock proteins (*HSP*) genes in buffalo mammary epithelial cells during heat stress condition. The total RNA extracted from individual MECs samples exhibited high mean (±SEM) *A*260/280 ratio of 2.06 ± 0.014, indicating high RNA quality from each sample. The qPCR performance for each gene in terms of coefficient of determination (R^2), and efficiency of amplification ($E = 10^{-1/\text{slope}}$) on the basis of slope of six-point standard curve are summarized in Table 1. The single peak observed for melting peak analysis indicated specific amplification for each gene under investigation.

For accurate normalization of target gene expression data, three most stable expressed genes *viz*; ribosomal protein genes (*RPL4* and *RPS23)* and eukaryotic translation elongation gene (*EEF1A)* identified in one of our previous studies (Kapila et al., 2013) were selected as

Table 1: Gene name, primer sequences, annealing temperature (T_a), slope, PCR efficiency and regression coefficient for the target genes under present investigation.

Gene Symbol	Description	Biological function	Primers 5'-3' (forward, reverse)	T_a (°C)	Slope	PCR Efficiency (%)	R^2value
HSPB1/ HSP27	Heat shock protein beta-1/ 27	Protects cells from cytotoxic effects of protein misfolding	TACATTTCCCGTTGCTTCAC GGACAGAGAGGAGGAGAC	60	-3.124	108	0.993
HSP40	Heat shock protein 40	Stimulate ATPase activity of HSP70.	AACACAACGGGTATGGT AGCCAGGATCAGCCTTC	60	-3.239	103	0.966
HSP60	Heat shock protein 60	Transportation and refolding of proteins	CGACAACTTCTGCTGTTGTTA ATGATGCTATGCTTGGAGAT	60	-3.389	97	0.972
HSP70	Heat shock protein 70	For protein folding and help to protect cells from stress.	AACATGAAGAGCGCCGTGGAGG GTTACACACCTGCTCCAGCTCC	60	-3.03	113	0.993
HSP90	Heat shock protein 90	Signal transduction, protein folding, Protein degradation, and morphologic evolution.	CTGTCATCAGCAGTGGG ACATGCCAACAGGATCTAC	60	-3.009	114	0.992

HKGs. Briefly, a total of 16 genes were evaluated as potential HKGs in buffalo MECs during heat stress condition. All the three different algorithms (geNorm, Normfinder and BestKeeper) employed to identify best suitable HKGs showed almost similar trend stability ranking. All the studied genes performed well displaying M values (stability values) below the cut off limit of 1.5. Based on the expression stability criteria, the genes were ranked from the most stable (lowest *M* value) to the least stable (highest *M* value): *RPL4, EEF1A1 > RPS23 > GTP > UXT > RPS*9 *> RPS15A > HMBS > B2M > HPRT1 > UBC > RPS18 > GAPDH > ACTB > RPL22> A2M* (Fig. 1). Therefore, for normalization of HSP expression data, geometric mean of *RPL4*, *RPS23* and *EEF1A1* was utilized in the present study for getting the accurate analysis.

The mRNA expression pattern of individual HSP gene in response to heat stress is depicted in Fig. 2. The qPCR expression data showed immediate increase in mRNA level of all the analyzed HSPs in response to *in vitro* heat stress. Each member of the studied molecular chaperons (HSP family) responded well within 30 min and most of them remained elevated till 12h post heat stress. Most of them showed maximum increase in mRNA expression at 2h-4h post heat stress and at later stages of recovery (16h-48h post heat stress), the expression level declined to the level of unstressed MECs (Fig.2). The increase in expression of HSP genes suggested the responsiveness of buffalo MECs to heat stress *in vitro*. The induction of chaperone activity in buffalo MECs indicated the presence of thermoregulatory mechanism during early stages of cellular response to heat stress.

Among different HSP genes, *HSP70* was most responsive showing higher expression level during 30m-8h time points post heat stress. The maximum induction (72.54 fold) in its expression was observed at 3h-4h post heat stress in comparison to unstressed MECs. Further, *HSP70* mRNA remained elevated for 8h after heat stress, before returning to the basal level (Fig.2). Similar to the expression pattern shown by *HSP70*, the maximum expression for *HSP40* was observed at 2h-4h post heat stress with 20.45 fold higher expression in comparison to unstressed MECs. The *HSP40* gene was found to be second most abundantly expressed HSP in the present study.

Similar to *HSP70* and *HSP40*, *HSP60* and *HSP90* mRNAs showed maximum induction at 4h post heat stress with 1.97 and 3.11 fold increases, respectively. In contrast, *HSP27* mRNA showed maximum induction at 16h post heat stress with a fold change of 8.20. The increase in mRNA level of *HSP27*, *HSP60* and *HSP90* genes though was relatively low at different time point post heat stress in comparison to *HSP70* and *HSP40*. The ranking order of HSP genes from higher to lower abundance was; *HSP70>HSP40>HSP27>HSP90>HSP60.* Amongst different HSP, *HSP70* transcript was found to be the most abundantly inducible form synthesized in buffalo MECs during heat stress. *HSP27*, *HSP60* and *HSP90* genes though were moderately induced yet their expression remained elevated at longer period of time post heat stress in comparison to strongly induced *HSP70* and *HSP40* mRNA (Fig. 2). The higher expression of various HSP genes throughout the time course indicated their prolonged chaperon activity in helping MECs to escape from the negative impact of heat stress. The increased synthesis of HSP during variety of stressful conditions, including heat, toxins, oxidative stress, and glucose deprivation is a well known mechanism developed by all organisms to protect themselves from cellular stress. Both *in vivo* and *in vitro* studies have shown that various stressors transiently increase the production of HSPs as protection against stress (Ritossa, 1962). To the best of our knowledge, this is the first report to present the expression pattern of different HSP transcripts in MECs of buffaloes under *in vitro* heat shock condition. The reason for selecting five major heat shock protein genes (*HSP70*, *HSP90*, *HSP60*, *HSP40*, *HSP27*) for the present analysis was due to their primary role as molecular chaperons that ensures the correct protein folding and apoptosis regulation during physiological stressful conditions (Collier et al., 2008).

Our findings were in accordance with previous studies where heat stress led to induction of HSP genes and reduction of cellular growth and downregulation of genes associated with cellular metabolism on the other hand (Collier et al., 2006, 2008). Further, it was reported that heat shock remarkably impacted the secretion of milk

protein and heat tolerance ability of mammary epithelial cells (Hu et al., 2011). Our findings were also in accordance with induction of expression of HSPs in other cell types (leukocytes/lymphocytes) due to heat stress (Agnew and Colditz, 2008; Guerriero and Raynes, 1990; Dangi et al., 2012).

Conclusion

This paper presents the expression pattern of *HSP27*, *HSP40*, *HSP70*, *HSP60*, and *HSP90* genes in heat stressed buffalo MECs. *HSP70* was identified as the most predominant form of *HSP* transcripts induced in buffalo MECs due to heat stress. The study has provided the initial evidence to suggest that mRNA of five HSP genes have varied level of induction in expression in buffalo MECs. The responsiveness of buffalo MECs to heat stress in the present study clearly suggested its suitability as a model to understand the modulation of buffalo mammary gland expression signature in response to environmental heat load. In future, such studies may be extended in evaluating the impact of hyperthermia and other physiological stressors in tissue damage and related gene regulation studies to understand buffalo mammary functions.

Acknowledgments

The work was supported by Indian Council of Agricultural Research, New Delhi, under National Fellow project.

References

Anand V., Dogra N., Singh S., Kumar S.N., Jena M.K., Malakar D., Dang A.K., Mishra B.P., Mukhopadhyay T.K., Kaushik J.K., Mohanty A.K. (2012). Establishment and Characterization of a Buffalo (Bubalus bubalis) Mammary Epithelial Cell Line. *PLoS ONE* 7:7.

Andersen C.L., Jensen J.L., Orntoft T.F. (2004). Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization applied to bladder and colon cancer data sets. *Cancer Res* 64:5245-5250.

Agnew L.A., Colditz .IG. (2008). Development of a method of measuring cellular stress in cattle and sheep. *Veterinary Immunology and Immunopathology* 123:197–204.

Bond T.E. (1969). Livestock environment research review. Annu Meet of the ASAE, Pacific Coast, Region, Phoenix, AR.

Collier R.J., Stiening C.M., Pollard B.C., VanBaale M.J., Baumgard L.H., Gentry P.C., Coussens P.M. (2006). Use of gene expression microarrays for evaluating environmental stress tolerance at the cellular level in cattle. *J Anim Sci* 84(E Suppl):E1–E13.

Collier R.J., Collier J.L., Rhoads P. and Baumgard L.H. (2008). Invited Review: Genes involved in the bovine heat stress response. *J Dairy Sci* 91:445-454.

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., Taru Sharma G., Sarkar M. (2012). Expression profile of HSP genes during different seasons in goats (Capra hircus). *Trop Anim Health Prod*. 44(8):1905-12 doi: 10.1007/s11250-012-0155-8.

DeMeester S., Buchman T., Qiu Y., Jacob A., Dunnigan K., Hotchkiss R., Karl I., Cobb J. (1997). Heat shock induces IκB-α and prevents stress-induced endothelial cell apoptosis. *Arch Surg* 132:1283–1287.

Du J., He-Shuang D.I., Liang G.U.O., Zhong-Hao L.I., Ya-Fei C.A.I., Gen-Lin WANG (2006). The effect of high temperature on mammary epithelial cells proliferation and apoptosis. *Acta zoological Sinica* 52:959-965.

Du J., Di H.S., Wang G.L. (2007). Establishment of a bovine epithelial mammary cell line and its ultrastructural changes when exposed to heat stress. *Sheng Wu Gong Cheng Xue Bao* 23:471–476.

Fuquay J.W. (1981). Heat stress as it affects animal

Garcia-Bermejo L., Vilaboa N., Perez C., Galan A., De Blas E., Aller P. (1997). Modulation of heat-shock protein 70 (HSP70) gene expression by sodium butyrate in U-937 promonocytic cells: relationships with differentiation and apoptosis. *Exp Cell Res* 236:268–274.

Guerriero V. Jr., Raynes D.A. (1990). Synthesis of heat stress proteins in lymphocytes from livestock. *J Anim Sci*. 68(9):2779-83.

Hu H., Wang J., Bu D., Wei H., Zhou L., et al (2009). In Vitro Culture and Characterization of a Mammary Epithelial Cell Line from Chinese Holstein Dairy Cow. *Plos One* 4(11).

Hu Han., Wang J.Q., Li F.D., Bu D.P., Zhou L.Y., (2011). Responses of cultured bovine mammary epithelial cells to heat stress. *Journal of Agricultural Biotechnology* 19 (2):287-293.

Huynh H.T., Robitaille G., Turner J.D. (1991). Establishment of bovine mammary epithelial cells (MAC-T): an in vitro model for bovine lactation. *Exp Cell Res* 197:191–199.

Kapila N., Kishore A., Sodhi M., Sharma A., Kumar P., Mohanty AK., Jerath T., Mukesh M. (2012). Identification of appropriate reference genes for qRT-PCR analysis of heat stressed mammary epithelial cells in riverine buffaloes (*Bubalus bubalis*). *ISRN Biotech http://www.isrn.com/journals/biotechnology/aip/735053/*

Johnson H.D. (1965). Environmental temperature and lactation with special reference to cattle. *International J Biom* 9:103-116.

Juan D., He-Shuang D., Liang G., Zhong-H. L., GenL.W. (2008). Hyperthermia causes bovine mammary epithelial cell death by a mitochondrial-induced pathway. *Journal of Therm Biol* 33:37–47.

Lauzon K., Zhao X., Bouetard A., Delbecchl L., Paquette B., Lacasse P. (2005). Antioxidants to prevent bovine neutrophil-induced mammary epithelial cell damage. *J Dairy Sci* 88:4295–4303.

Li J.X., Zhang Y., Ma L.B., Sun J.H., Yin B.Y. (2009). Isolation and culture of bovine mammary epithelial stem cells. *J Vet Med Sci* 71:15–19.

Liu H.Y., Yang J.Y., Wu H.H., Wu Y.M., Liu J.X. (2007) .Effects of methionine and its ratio to lysine on expression of caseinαs1 in cultured bovine mammary epithelial cells. *J Anim Feed Sci* 16:330–334.

Livak K.J., Schmittgen T.D. (2001). Analysis of relative gene expression data using realtime quantitative PCR and the 2ΔΔC (T) Method. *Methods* 25:402–408.

Marai, I.F.M. and Habeeb, A.A.M. (2010). Buffalo biological functions as affected by heat stress - a review. *Livestock Science* 127: 89-109.

McDowell R.E. (1966). The role of physiology in animal production for tropical and subtropical areas. *World Rev Anim Prod* 1:39.

Mosser D., Caron A., Bourget L., Denis-Larose C., Massie B. (1997). Role of the human heat shock protein hsp70 in protection against stress-induced apoptosis. *Mol Cell Biol* 17:5317–5327.

Pfaffl M.W., Tichopad A., Prgomet C., Neuvians T.P. (2004). Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations. *Biotechnol Lett* 26:509–515.

Ritossa F. (1962). A new puffing pattern induced by heat shock and DNP in Drosophila. *Experientia* 18:571–573.

Thatcher W.W. (1974). Effects of season, climate and temperature on reproduction and lactation. *J Dairy Sci* 57:360.

Vandesompele J., Preter K.D., Pattyn F., Poppe B., Roy N.V., Paepe A.D., Speleman F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3 7 Research 0034.

Zavizion B., Gorewit R.C., Politis I. (1995). Subcloning the MAC-T bovine mammary epithelial cell line: morphology, growth properties, and cytogenetic analysis of clonal cells. *J Dairy Sci* 78:515–527.

Zhao K., Liu H.Y., Zhou M.M., Liu J.X. (2010). Establishment and characterization of a lactating bovine mammary epithelial cell model for the study of milk synthesis. *Cell Biol Inter* 34: 717–721.