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Differential expression of heat shock proteins in tissues of riverine buffaloes

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ABSTRACT

In this study, an effort was made to study tissue-specific expression of some of the major heat shock protein (*HSP*) genes in riverine buffaloes. Samples (30) comprising 5 each of kidney, liver, muscle, heart, mammary gland and PBMCs were utilized for expression analysis under no heat shock condition. Amongst *HSPs*, *HSP27* mRNA showed maximum expression in all the analyzed 5 tissues, viz. heart, kidney, liver, muscle and mammary gland, indicating this to be the most abundant form. However, in comparisons to tissues, *HSP27* expression was low in PBMCs. On the other hand, *HSP40* transcript was expressed at higher level in PBMCs while *HSP60* and *HSP90* transcripts were found highly expressed in mammary gland. The expression of *HSP70* mRNA was highest in muscle, however *HSP70* mRNA level was prominently high in all other tissues. The study helped to generate base line expression data on major *HSP* genes in different buffalo tissues. In future, the information presented here would be useful in evaluating the tissue specific response to any physiological and thermal stressors in buffaloes.

Key words: Buffalo, Gene expression, Heat shock protein, Real-time PCR, Tissues

Heat shock proteins (HSPs) have been identified as major proteins induced during cellular responses to various stresses such as heat shock and oxidative stress (Lindquist and Craig 1988, 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. 2004). *HSPs* get accumulated to high levels in stressed cells and could remain elevated for a long period of time (Georgopoulos and Welch 1993). However, besides inducible *HSPs*, several *HSPs* are subsequently shown to express in the absence of any stress and play important roles during normal cell growth and differentiation (Christians *et al.* 2003, Meistertzheim *et al.* 2009). The *HSP* genes known to be highly conserved are well characterized in wide range of organisms.

In livestock, few isolated studies have been carried out on heat-stress associated genes/transcripts (Moran *et al.* 2006, Lacetera *et al.* 2006, Collier *et al.* 2008). However, to date, expression kinetics of heat stress associated transcripts is poorly understood in riverine buffalo (*Bubalus bubalis*). Heat stress phenomenon has become a major issue in the era of climate change. The riverine buffalo suffer a great deal to heat stress, affecting both its reproductive and productive performance. However, unlike other organisms, very little information is available in this species about the expression and tissue distribution of the heat shock protein genes (*HSPs*), which are known for regulating cellular homeostasis and survival during hyperthermia. The current study was therefore conducted to determine the basal expression level of 5 important heat shock protein genes (*HSP27, HSP40, HSP60, HSP70* and *HSP90*) across different tissues and peripheral blood mononuclear cells (PBMC) in buffaloes.

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MATERIALS AND METHODS

Collection of samples: Tissue samples (kidney, liver, muscle, heart and mammary gland) from 5 adult riverine buffaloes were collected from an abattoir (Gajipur, New Delhi) and immediately transferred to laboratory in RNA later solution. The collections were made in March-April. For PBMC, whole blood samples were collected from the same 5 animals and processed immediately for PBMC isolation using density gradient centrifugation. Briefly, the blood diluted in phosphate buffered saline was gently overlaid on Histopaque-1077 in ratio of 1: 1 and centrifuged. The mononuclear cells were recovered carefully and washed twice in PBS. The residual red blood cell contamination was eliminated by treating the cells with RBC lysis buffer. The viability of PBMC was measured manually using the Trypan blue dye exclusion method.

Total RNA isolation and cDNA synthesis: Total RNA was extracted from 30 samples using using ice-cold Trizol. To remove the traces of genomic DNA, RNeasy Mini Kit

columns along with on coloum digestion by RNAse free DNase enzyme were used. Total RNA concentration and purity was measured by spectrophotometer. The purity of RNA (A_{260}/A_{280}) for all samples was above 1.9. All the extracted RNA were stored at –80°C and utilized within 1 month. cDNA was synthesized using 100 ng RNA, 1 µl dT_{12-18}, 1 µl 10 mmol/L dNTP mix, 1 µl random primers, 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 composed of 4.5 µl 5X First-Strand buffer, 1 µl 0.1 M DTT, 0.25 µl (50 U) of SuperScript™ III RT, and 0.25 µl of RNase inhibitor (10 U,) was added. The reaction was performed in an Eppendorf Gradient cycler using the programme: 25°C for 5 min, 50°C for 60 min and 70°C for 15 min. Before use, the cDNA was diluted 1: 4 (v: v) with DNase/RNase free water.

Quantification of HSP genes expression by real-time qRT-PCR: The expression of 5 *HSP* family genes namely, *HSP27, HSP40, HSP60, HSP70* and *HSP90* in different samples was measured by real-time quantitative reverse transcription PCR (real-time *qRT-PCR)*. The primers details for *HSP* genes and 2 housekeeping genes, *ACTB* and *GAPDH* are given in Table 1. Prior to *qRT-PCR*, primers specificity was confirmed in a 20µL PCR reaction using the same protocol described for *qRT-PCR* except for the final dissociation protocol. PCR product was run in 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. Each reaction was performed using 4 µl diluted cDNA combined with 6 µl of a mixture composed of 5 µl 2 X LightCylcer 480 SYBR Green I master mix, 0.4 µl each of 10 µM forward and reverse primers, and 0.2 µl DNase/RNase free water in a 96 well white plate. qPCR was conducted on cDNA samples with 18 primer-pairs. For each gene, samples were run in duplicate (technical replicates) along with 6 point relative standard curve plus the non-template control (NTC). The reactions were performed in a LightCylcer 480 instrument using the amplification conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 sec at 95°C (denaturation) and 1 min at 60°C (annealing + extension). The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures of 95°C for 15 sec plus 65 °C for 15 sec. The *qRT-PCR* expression data for each ICG was extracted in the form of crossing points. The data was acquired using the 'second derivative maximum' method as computed by the LightCycler Software 3.5 and subjected for subsequent analysis.

Data analysis: In the current study, the analysis of mRNA expression data across different samples was based on C_T (cycle threshold) values. The C_T values of each of the target gene were subtracted from the arithmetic mean of C_T values of the 2 HKGs to calculate ΔC_T. To compare the differences in the expression levels of different genes in same tissue or one gene in different tissues, the ΔC_T values were analyzed using one-way ANOVA test followed by Tukey's multiple comparison tests and P value of <0.05 was considered statistically significant. All data are presented as the mean ±SEM.

RESULTS AND DISCUSSION

The current study describes the basal expression pattern of *HSP27, HSP40, HSP60, HSP70,* and *HSP90* genes under no heat shock condition in heart, kidney, liver, muscle, mammary gland and PBMCs of buffaloes. The RNA extracted from each sample exhibited high purity as determined by $A_{260/280}$ ratio of 2.010 ± 0.013. The melt curve analysis indicated specificity of each qPCR reaction. The parameters that were used to determine the qPCR amplification efficiency ($E=10^{-1}$/slope) and coefficient of determination (R^2) of the analyzed genes based on 6-point standard curve are mentioned in Table 1. The amplification of the genomic DNA during *qRT-PCR* reaction was taken care by employing DNAse treatment of total RNA. The use of both random hexamers and oligo(dTs) during reverse transcription reaction for all the samples further helped in reducing the chances of data variation. The expression data was normalized using 2 of the most commonly used reference genes (*GAPDH* and *ACTB*). Detection of each of the *HSP* transcripts in all the studied buffalo tissue samples indicated their constitutive expression.

Distribution of HSP mRNA in different tissues: The basal expression pattern of individual *HSP* gene in different tissues is plotted in Fig. 1A - E. *HSP27* mRNA showed maximum expression in all the analyzed 5 tissues, viz. heart, kidney, liver, muscle and mammary gland with no

Table 1. The primers used in qRT-PCR, annealing temperature (T_a), slope, PCR efficiency and regression coefficient (R^2value) for each of the studied genes

Gene Symbol	Primers 5'-3' (forward, reverse)	T_a (°C)	Slope efficiency (%)	PCR	R^2value
HSP27	TACATTTCCCGTTGCTTCACGGACAGAGAGGAGGAGAC	60	–3.211	104.847	0.999
HSP40	AGCCAGGATCAGCCTTC; AACACAACGGGTATGGT	60	–3.438	95.374	0.981
HSP60	CGACAACTTCTGCTGTTGTTA; ATGATGCTATGCTTGGAGAT	60	–3.443	95.184	0.996
HSP70	AACATGAAGAGCGCCGTGGAGG; GTTACACACCTGCTCCAGCTCC	60	–3.038	113.385	0.994
HSP90	CTGTCATCAGCAGTGGG; ACATGCCAACAGGATCTAC	60	–3.097	110.326	0.995
GAPDH	TGGAAAGGCCATCACCATCT; CCCACTTGATGTTGGCAG	60	–3.361	98.395	0.999
ACTB	GCGTGGCTACAGCTTCACC; TTGATGTCACGGACGATTTC	60	–3.488	93.507	0.999

significant difference (P > 0.05; Fig. 1A). However, in comparisons to different tissues, PBMC contained significantly less amount of *HSP27* transcript (P < 0.05). Instead, *HSP40* mRNA was detected at highest level in PBMC followed by mammary gland, kidney, liver, muscle and heart (Fig. 1B). Muscle and heart contained almost similar amount of *HSP40* mRNA that were approximately 2-fold less than PBMC. Similarly, kidney and muscle showed similar amount of *HSP40* transcript that were 1.5-fold less than its amount detected in PBMC. *HSP60* transcript was detected in highest amount in mammary gland and the expression was significantly different than that observed in PBMC and heart (P < 0.05, Fig. 1C). Similarly, kidney and liver also contained significantly greater amounts of *HSP60* transcript in comparison to PBMC and heart (P < 0.05). Like *HSP60*, *HSP90* mRNA was detected at highest level in mammary gland (Fig.1E). However, the difference in *HSP90* mRNA level among the different tissues was nonsignificant (P > 0.05). *HSP70* mRNA level was prominently high in all tissues with marginal differences (Fig.1D). Comparatively, *HSP70* mRNA was highest in muscle and lowest in liver and the observed difference was statistically significant (P < 0.05).

Tissue wise mRNA levels of different HSP genes: Additionally, the distribution of individual *HSP* mRNA was analyzed within each tissue and tissue specific data is plotted in Fig. 2A -E. In heart tissue, *HSP27* mRNA level was most pronounced (Fig. 2A). For other *HSPs*, moderate to low mRNA level was observed with *HSP40* showing least abundance. *HSP40* mRNA was about 7.5-fold down regulated than that of *HSP27*, while transcripts of *HSP60*, *HSP70*, and *HSP90* were 5.5-, 2.75-, and 2.9- fold less, respectively. In heart, the genes were ranked from higher to lower abundance in the following order; *HSP27>HSP70>HSP90>HSP60>HSP40*.

Like heart, in kidney also *HSP27* was predominant mRNA species (Fig. 2B). The other *HSPs* were present in moderate to low abundance. The transcripts of *HSP40*, *HSP60, HSP70, HSP90* were about 3.8-, 2.0-, 2.2-, and 3.3-fold less, respectively, than that of *HSP27* mRNA. In kidney, the ranking order of genes from higher to lower abundance was; *HSP27> HSP60> HSP70> HSP90> HSP40*.

Similar to heart and kidney, *HSP27* transcript was predominant mRNA species in liver as well (Fig. 2C). The transcripts of *HSP40*, *HSP60, HSP70, HSP90* were about 4.3-, 2.2-, 3.8-, and 4- fold less, respectively, than that of *HSP27* mRNA. In liver, the ranking order of genes from higher to lower abundance was; *HSP27> HSP60> HSP70> HSP40> HSP90*.

In muscle also, *HSP27* transcript was observed in predominant form (Fig. 2D). The transcripts of *HSP40*, *HSP60, HSP70,* and *HSP90* were about 8.3-, 6.2-, 2.5-, and 2.5- fold less, respectively, than that of *HSP27* mRNA. In muscle, the ranking order of genes from higher to lower abundance was; *HSP27> HSP70> HSP90> HSP60> HSP40.*

Similar to other buffalo tissues, in mammary gland as well, *HSP27* transcript was observed to be the most dominant form (Fig. 2E). The transcripts of *HSP40*, *HSP60, HSP70, HSP90* were about 3.5-, 6.2-, 2.5-, and 2.5- fold

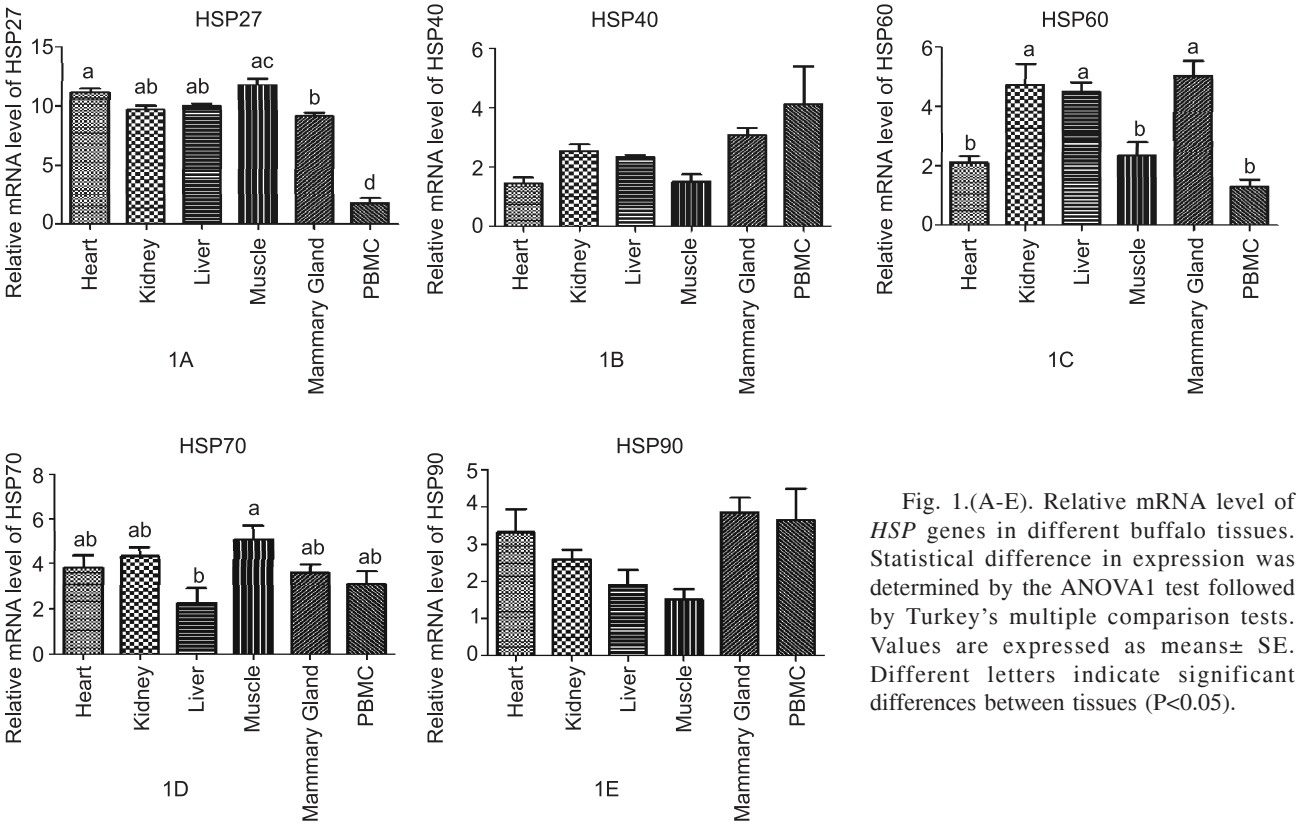

Fig. 1.(A-E). Relative mRNA level of *HSP* genes in different buffalo tissues. Statistical difference in expression was determined by the ANOVA1 test followed by Turkey's multiple comparison tests. Values are expressed as means± SE. Different letters indicate significant differences between tissues (P<0.05).

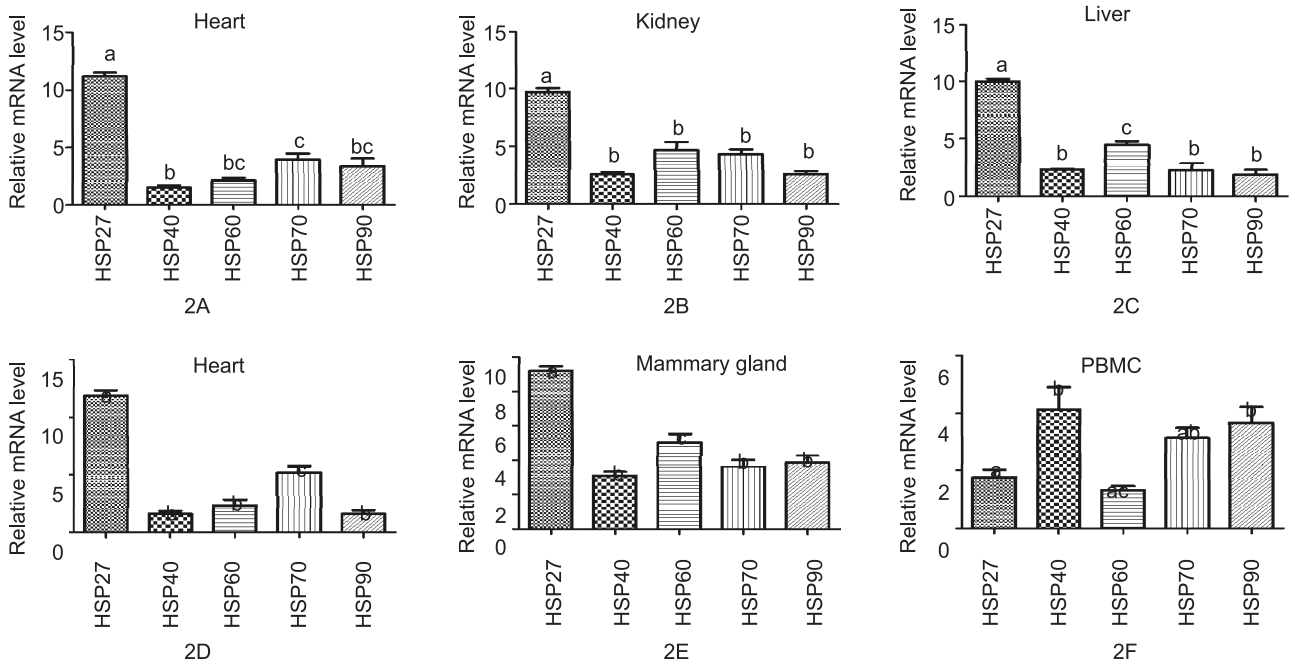

Fig. 2(A-F). Tissue wise mRNA levels of different *HSP* genes. Statistical difference in expression was determined by the ANOVA1 test followed by Turkey's multiple comparison tests. Values are expressed as means± SE. Different letters indicate significant differences between tissues (P<0.05).

less, respectively, than that of *HSP27* mRNA. In mammary gland, the ranking order of genes from higher to lower abundance was; *HSP27*> *HSP60*> *HSP90*> *HSP70*> *HSP40.*

Interestingly, in PBMC instead of *HSP27, HSP40* mRNA was the predominant mRNA species (Fig. 2F). Taken together, these results clearly revealed *HSP27* to be the most abundant form of the examined *HSP* genes in all the tissues under unstressed condition, whereas *HSP40* transcript was least abundant in majority of the tissues. In liver, *HSP40* transcript expression was marginally higher to the least abundant *HSP90* mRNA. The detection of each *HSP* transcripts albeit at varied levels indicated their tissue specific physiological role in buffaloes.

This is the first report to describe the basal expression profile of different *HSP* transcripts in various buffalo tissues under no heat shock condition. The reason for selecting 5 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 (Hartl and M. Hayer-Hartl 2002, Kregel 2002). It is also noteworthy that though heat shock response is an evolutionary conserved mechanism, different eukaryotic cells/tissues may vary in their inherent ability to induce *HSP* synthesis during heat shock. The increased synthesis of *HSP* that accumulate to high levels in a variety of stressful conditions, including heat, toxins, oxidative stress, and glucose deprivation (Srivastava 2002) is a well-known mechanism developed by all organisms to protect themselves from cellular stress. While most of genes encoding *HSPs* are well characterized in different species especially model organisms, limited knowledge is available in livestock species, especially buffaloes. The role of these constitutively expressed *HSPs* are not completely understood in livestock species, however their diverse expression in different tissues could be related to the mechanism involved in physiological cellular processes like maintaining appropriate cell number and balance between proliferation, differentiation and apoptosis. In past, several reports showed the constitutive expression of *HSP* family transcripts in cells/tissues in the absence of stress (Sarge and Cullen 1997, Meistertzheim *et al.* 2009, Tucker *et al.* 2009).

Amongst genes examined in this study, *HSP*27 was found to be most dominant transcript expressed in different tissues (Fig. 1E). *HSP27* also known as *HSPB1* is a mammalian *HSP* family member that is known to be expressed in a variety of tissues in the presence or absence of stress (Hickey *et al.* 1986, Concannon *et al.* 2003). Its function as a molecular chaperone to aid in refolding of inactive proteins under normal conditions is well reported. It is also associated with cytoskeleton and actin microfilaments regulation and reported to be over expressed during exposure to stressors such as hyperthermia, oxidants or cytochalasin D, transportation (Concannon *et al.* 2003, Zhang *et al.* 2011). The ability of this gene to protect cultured cells from thermal injury has been reported by Mao *et al.* (2005). Recently, the changes in its expression level are associated with the protection of heart tissue in pigs (Zhang *et al.* 2011). It is also linked with inhibition of apoptosis and as biomarker for numerous diseases (Brown

et al. 2007, Vidyasagar *et al.* 2008, Vidyasagar *et al.* 2012). In the absence of any stress, its constitutive expression was equally observed in several tissues, including all types of muscle cell (Tucker *et al.* 2009, Dillmann 1999, Benndorf and Welsh 2004), heart (Tucker *et al.* 2009) and in many non-stressed cultured cells (Arrigo 1990a, Arrigo 1990b, Preville *et al.* 1996). Similar to the studies reported in zebrafish (Tucker *et al.* 2009, Marvin *et al.* 2008), the present data also showed higher abundance of *HSP27* in muscle and heart tissues (Fig. 1E). However, it is noteworthy that McComb and Spurlock (1997) could not find expression of *HSP27* in normal porcine heart, lung, liver, brain, muscle, small and large intestine. Their failure to detect *HSP27* in pig tissues could be due to lack of homology of used monoclonal antibodies with the porcine *HSP27* protein or its very low level expression. The constitutive and highly expressed *HSP27* mRNA in different buffalo tissues suggests its putative role in defense mechanism against cell damage and also as housekeeping gene. In future, it would be interesting to identify transcription factors and regulatory elements of this gene for better understanding of regulation of this gene in buffalo during stressed and unstressed period.

Similar to *HSP27*, *HSP70* transcript abundance was highest in buffalo muscle tissue. This could be attributed to increased metabolic demands and protein turnover associated with increased muscular activity. Its higher level in buffalo muscle and other tissues may also be associated to putative mechanism for protecting buffalo tissues against any damaging stressors including heat or acute inflammation. Poltronieri *et al.* (2007) also showed ubiquitous expression of inducible *HSP70* mRNA in liver, brain, muscle, gills, kidney, gonads, heart, spleen and skin of sea bass fish. They also detected highest levels of inducible *HSP70* mRNA in the muscle tissue. Few other studies have also confirmed the basal expression of the stress-inducible *HSP70* mRNA in different tissues e.g., neuron of normal rabbit brain (Foster and Brown 1996), during development of eye lens in zebrafish (Blechinger *et al.* 2002), normal follicles in cattle (Velázquez *et al.* 2011), normal heart, liver, brain and skeletal muscle in turtle (Scott *et al.* 2003). Based on current understanding of *HSP70* function, it is noteworthy that tissues expressing the highest level of inducible *HSP70* are expected to be relatively resistant to heat stress. Our current result indicated that buffalo muscle tissue should suffer less damage while liver showing lowest level of *HSP70* expression should be the most affected tissue to thermal stress. In future, these results may lay the basis for systematic examination of inter-tissue variability in thermal response/sensitivity in buffaloes.

We noted higher expression of *HSP60* mRNA in mammary gland followed by kidney, liver, heart, muscle and PBMC. Few other studies, have also reported about its constitutive expression in unstressed eukaryotic cells (Zhao *et al.* 2002, Yang *et al.* 2009). *HSP60* is known to be one of the most important molecular chaperons under various stressful conditions (Oksala *et al.* 2006). A number of studies indicated that *HSP60* localizes in mitochondria and plays important role in the folding, unfolding and translocation of mitochondrial and cellular proteins (Habich and Burkart 2007). The differential expression of *HSP60* mRNA like other *HSPs* observed in the current study indicated the presence of tissue dependent cytoprotection of *HSP60* in buffaloes.

Unlike most of the other tissues wherein *HSP40* mRNA was least abundant, its expression was highest in PBMC. It should be noted that *HSP40* generally functions as a co-chaperone/cofactor of mammalian *HSP70* and the complex is involved in the restoring of protein confirmation after heat shock (Welch 1990, Morimoto *et al.* 1998). The pronounced *HSP40* mRNA expression in PBMC as observed in present study could be linked to its role in stimulating PBMC to secrete cytokines during stressful/or diseased condition. Similarly, the immunological effect of *HSP40* in modulating humoral and cellular immune response as suggested by Tukaj *et al.* (2010) may also be true for buffaloes.

In contrary to *HSP27* and *HSP70, HSP90* expression was lowest in muscle and highest in mammary gland (Fig.1B). Its predominance in mammary gland was similar to *HSP60* transcript, which was also the most dominant form in mammary tissue. The mammary specific function of *HSP90* could be inferred from the work of Watanabe *et al.* (1997), wherein, its role during lactation and mammary gland development was reported. *HSP90* is known to act as co-chaperon with other *HSPs* such as the *HSP70* family proteins (Pratt and Toft 2003). Like other members of *HSP* family, it is also a highly conserved and abundant protein involved in protective mechanism against heat shock stress. Further, *HSP90* is known to be involved in several signal transduction and cellular regulatory pathways (Zhang and Burrows 2004, Brown *et al.* 2007).

In conclusion, this paper presents the tissue specific basal expression pattern of *HSP27, HSP40*, *HSP70*, *HSP60*, and *HSP90* in heart, kidney, liver, muscle, mammary gland and PBMC of buffalo. *HSP27* is identified as the most predominant form of *HSP* transcripts expressed across different buffalo tissues. The study thus provides initial evidence to suggest that the mRNA of five *HSP* genes have constitutive albeit varied expression level in normal buffalo tissues. The detection of these endogenously expressed *HSP* genes in non-stressed buffalo tissues are not only be linked to their normal intracellular functions such as regulation of protein folding, assembly and translocation across membranes but can also influence the heat shock response and sensitivities of different buffalo tissues to stressful situation. Further, the observed differences in gene expression patterns of *HSPs* could also reflect the inherent variations in tissue organization and tissue-dependent cyto-protection of different *HSP*s. In future, such studies may be useful in evaluating the impact of physiological and thermal stressors in tissue damage and related gene regulation studies in buffaloes.

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