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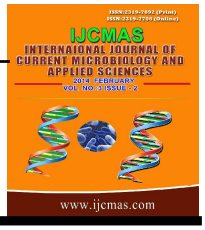

Original Research Article

Heat and cold stress enhances the expression of heat shock protein 70, heat shock transcription factor 1 and cytokines (IL-12, TNF-α and GMCSF) in buffaloes

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A B S T R A C T

In this study the differential expression of heat shock protein 70 (HSP70), heat shock transcription factor 1(HSF1) and cytokines; interleukin-12 (IL-12), tumour necrosis factor-α (TNF-α) and granulocyte macrophage colony stimulating factor (GMCSF), under heat and cold stress was investigated in buffaloes. The relative expressions were studied in Real Time PCR using SYBR green chemistry of HSP70, HSF1, IL-12, TNF-α and GMCSF increased with heat or cold stress. A negative correlation between milk production and both HSP70 and HSF1 expression in summer and winter was observed. As the expression of both the genes increased to cope up with the stress there was a significant decrease in milk production because of the metabolic load. The greater expression of HSF1 than the expression of HSP70 and a significant positive correlation between the expression of both the genes in summer and winter indicated the need of the HSF1 trimmers for the expression of HSP70. The expression of HSP70 positively correlated with the expression of all the cytokines genes investigated in the study indicating the representative role of HSP70 as a cytoprotectant.

Keywords

IL-12; TNF-α ; GMCSF Cytokines; Heat and cold stress

Introduction

Indian dairy animals have low milk production during summer and autumn seasons, presumably due to environmental in losing heat and will decrease its heat production by lowering feed intake that results in lower production or growth.

Heat stress is caused by those factors that decrease heat transfer from an animal to its environment, which would include high air temperature, high air humidity, low air movement and thermal radiation load.

Cellular response to stress includes synthesis of proteins belonging to a subgroup of molecular chaperones called heat shock proteins (HSPs). HSPs are encoded by genes that are present in all cells, in all forms of life and in a variety of intracellular locations. Under stress HSP levels raise up to 15% or more of the total intracellular proteins as against their normal level of 5%. HSPs can be classified into 10 families each consisting of between one and five closely related proteins. The HSPs perform a multitude of housekeeping functions like folding and unfolding of proteins, assembly of multi-subunit complexes, thermotolerance and buffering the expression of mutations that are essential for cellular survival (Srivastava, 2002). Among the HSPs, HSP70 is the one that shows highest level of expression under stress. The acquisition of thermal tolerance is related to increased levels of the HSP70 protein. The 70-kDa HSP assists the folding of other proteins. It binds to nascent peptide chains on ribosomes, protecting the hydrophobic surface that would normally be exposed to solvent, thus preventing aberrant folding or aggregation, until the whole peptide chain is synthesized and proper folding occurs (Alberts et al. 2004). However, the role of HSP70 is not confined to chaperone function as it has been shown to induce cytokine production (Srivastava, 2002; Asea *et al*.,2002).

Heat shock transcription factor 1 (HSF1) is recognized as the primary transcription factor responsible for the transcriptional response to heat stress in mammalian cells. HSF1 provides a powerful tool for examination of the role of HSPs. The essential role of HSF1 in the up-regulation of HSPs, conferring the cytoprotection against stressors, was demonstrated by the observation that disruption of the activity of HSF1 leads to the loss of stress-induced HSP up-regulation and the emergence of cells that are sensitive to apoptosis (Morimoto 1991).

The present study was conducted to assess the differential expression of heat shock transcription factor 1 (HSF1), heat shock protein 70 (HSP70) and cytokines, interleukin-12 (IL-12), tumour necrosis factor-α (TNF-α) and granulocyte macrophage colony stimulating factor (GMCSF), under heat and cold stress in buffaloes.

Materials and Methods

Buffaloes and sampling

Fifteen buffaloes, being maintained at GADVASU livestock farm, Ludhiana, Punjab (India) that were in similar stage of lactation and parity and were under same management and nutritional regimen at the time of sampling were selected for the study. The 5-10 ml of blood was collected from each animal in 15 ml sterile tubes containing EDTA (EDTA, 1.5 mg/ml of blood). The tubes were kept in ice till further processing. The blood samples were collected at 3 intervals, both in summer [summer 1 (start of August), summer 2 (end of August) and summer 3 (mid September)] and winter [winter 1 (mid November), winter 2 (mid December) and winter 3 (start of January)] season, from each animal. The health of the animals was regularly monitored during the experimental period and the animals were found to be healthy and free

from any infectious diseases during the experimental period. The buffaloes were milked twice a day and milk yield of the individual buffalo was recorded at each milking on all test days. The Temperature Humidity Index (THI) is widely used to assess the impact of heat stress on dairy animals. The THI during the experimental period exceeded the critical comfort level of 72.

Erythrocyte lysis and isolation of leukocyte

Whole blood samples collected were stored at 2-8°C and processed within not more than 2 hrs. Leukocyte populations were obtained by standardized erythrocyte lysis for 5 min using an lysis buffer [containing 3.7 g disodium EDTA salt, 80.2 g NH_4Cl and 8.4 g $NaHCO_3$ per liter, and prepared in distilled H_2O treated with 0.1% diethyl pyrocarbonate (DEPC)] (Klevezas et al. 2000). Samples were centrifuged at 2500 rpm for 5 min. Lysis and centrifugation were repeated three times to obtain a blank pellet of cells.

RNA isolation from whole leukocytes

Leukocytes were processed immediately to obtain total RNA or were stored at -70°C for one month in 1 ml RNAlater buffer (Kraev et al., 2003). Cells were treated using TRI-reagent (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. Briefly, cells were lysed directly in the 15 ml tubes by passing them several times through a pipette to form a homogenous lysate and were allowed to stand for 5 min at room temperature. Afterwards, 0.2 ml chloroform per ml of TRI-reagent was added, shaken vigorously for 15 sec, kept for 15 min at room temperature and centrifuged at 12,000 rpm for 15 min at 4°C. The centrifugation separated the mixture into 3 phases. The aqueous phase was transferred to a fresh tube, 0.5 ml isopropanol per ml TRI-reagent used was added, incubated for 10 min at room temperature and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was removed and the RNA pellet was washed in 1 ml 75% ethanol per ml TRI-reagent, by centrifuging at 12,000 rpm for 5 min at 4°C. The RNA pellet was air-dried for 5 min and 50µl of water treated with 0.1% diethyl pyrocarbonate (DEPC) was added. Samples were kept on hot plates at 55-60^0C for 15 min to increase yield of RNA (Perez et al. 2007). The RNA samples were stored at -80^0C till further processing.

Quality of RNA

The concentration of recovered RNA was measured on nanodrop (Nanodrop 2000C, Thermo Scientific). The quality of RNA was acceptable if the ratio of optical density at 260 nm to that at 280 nm was >1.9. The quality of RNA was also checked on Experion automatic capillary electrophorosis system (Bio Rad)

First strand cDNA synthesis

The extracted RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit of QIAGEN (Cat.No.205311) according to manufacturer's instructions. Briefly, the purified RNA sample was incubated in genomic DNA Wipeout Buffer at 42°C for 2 minutes to effectively remove contaminating genomic DNA. After genomic DNA elimination, reverse transcription was done using a master mix prepared from Quantiscript Reverse Transcriptase, Quantiscript RT Buffer and RT Primer Mix. Quantiscript Reverse

Transcriptase has a high affinity for RNA and is optimized for efficient and sensitive cDNA synthesis from10 pg to 1 µg of RNA. The entire reaction takes place at 42°C and is then inactivated at 95°C. In contrast to other methods, additional steps for RNA denaturation, primer annealing, and RNase H digestion are not necessary.

Real Time PCR amplification

For quantitation of gene expression, the recommended concentration of 1µg of RNA template for each sample was taken in the study. The integrity of the cDNA was checked by amplification of 218 bp GAPDH gene fragment on 2% agarose gel. Relative quantitation was performed in triplicate using an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and reactions using a mixture of Power SYBR Green PCR Master Mix with ROX as a passive reference dye. Primer sets were designed using the Primer Express software program (Applied Biosystems, Foster City, CA). Real time-Polymerase chain reactions were performed according to standard protocols with the primers indicated in Table 1. Briefly, template cDNA was denatured at 94^0C – 10 min , followed by 40 cycles of 94^0C – 45 s; annealing temperature for 55^0C – 45 s, elongation at 72 ^{0}C for 45 s and final extension 72^0C – 10 min. After each PCR run, a melting curve analysis was performed for each sample to confirm that a single specific product was generated. Negative controls (NTC = no template control), comprised of the PCR reaction mix, without nucleic acid, were also run with each group of samples. For the test gene and endogenous control standards 10-fold serial dilutions were run in the study to estimate the efficiency of PCR Data were adjusted for PCR efficiency and starting template amount with the following equation (Abasht *et al*. 2009) :

$$\text{Adjusted Ct value} = \left\{ \frac{(40 - Ct_{\text{test gene}})}{[(40 - Ct_{\text{reference gene}}) / (40 - \text{Mean}_{\text{reference gene}})]} \right\} \times \left\{ \frac{\text{Slope}_{\text{test gene}}}{\text{Slope}_{\text{reference gene}}} \right\}$$

where $Ct_{\text{test gene}}$ = mean of the triplicate cycle threshold (Ct) values of the gene being tested; $Ct_{\text{reference gene}}$ = mean of the triplicate Ct value of the housekeeping reference gene 18sRNA; $\text{Mean}_{\text{reference gene}}$ = overall experimental mean of $Ct_{\text{reference gene}}$; $\text{Slope}_{\text{test gene}}$ = slope from 10-fold test gene standard regression equation; and $\text{Slope}_{\text{reference gene}}$ = slope from 10-fold reference gene standard regression equation (Abasht *et al*. 2008). The adjusted Ct value was corrected for assay-specific PCR efficiency with the use of the slope ratio ($\text{Slope}_{\text{test gene}}$ / $\text{Slope}_{\text{reference gene}}$) term of regression slope test gene regression slope of reference gene (Scott et al., 2008). The test gene standard was a PCR-amplified fragment containing the target segment, whereas the 18sRNA standard was a pooled sample of multiple RNA samples. The initial quantity of cDNA was normalized across samples by the correction of [$(40–Ct_{\text{reference gene}})$ / $(40−\text{Mean}_{\text{reference gene}})$] term (Kaiser *et al*. 2000; Abasht *et al*. 2009).

Statistical analysis

All analyses were performed using the GLM procedure of the JMP statistical program package (SAS Institute, 2009). Initial analysis revealed significance of both genetic line and treatment effects on expression level of all the test genes. Therefore, the data was separately analyzed for each genetic line considering treatment as fixed effect and PCR run as random effect. Multiple comparisons of least squares (LS) means were performed by Tukey's honestly statistical differences

test (SAS Institute, 2009). Differences were considered significant at $P \leq 0.05$.

Results and Discussion

The cellular response to various stress stimuli (heat or cold) activates a specific set of genes called heat shock genes which results in the selective synthesis of a group of proteins known as heat shock proteins (Luc et al. 2001; Christian et al. 2003). Heat-shock proteins (HSPs) are the most abundant and ubiquitous soluble intracellular proteins present in all cells in all forms of life and in a variety of intracellular locations. The expression of various HSPs confers tolerance to the cells under stress. The HSPs have strong cytoprotective effects, are involved in many regulatory pathways, and behave as molecular chaperones to protect the three dimensional structure of proteins of organisms which are exposed to environmental stress (Hightower 1991).The intracellular levels of HSPs were suggested as indicators of thermotolerance. The extreme (high or low) temperatures at GADVASU livestock farm triggered massive synthesis of heat shock proteins that fold heat-denatured proteins and block caspase dependent apoptosis, permitting repair and thwarting death.

HSF1 is the transcription factor necessary for transcription of HSP70 mRNA. The HSFs has been implicated to be important first responders during the onset of elevated cell temperature (Trinklein et al. 2004; Page et al. 2006). These transcription factors coordinate the cellular response to thermal stress and affect expression of a wide variety of genes including HSPs (Akerfelt et al. 2007). The physiological importance of HSF is exemplified by the evolutionary conservation between yeast (*Saccharomyces cerevisiae*), fruit flies (*Drosophila melanogaster*), vertebrates, and plants (Pirkkala et al. 2001). Although mammals express HSF1, 2 and 4. HSF1 is primarily responsible for inducing HSP gene expression during hyperthermia (Pirkkala et al. 2001). The current model of HSF1 transcriptional activity indicates that non stressed cells contain folded HSF1 monomers bound to HSP within the cytoplasm. Upon heat stimulus, the HSP dissociate from HSF1 monomers, which then unfold and bind to two other HSF1 monomers to form trimers before their nuclear translocation. Once in the nucleus, homotrimeric HSF1 binds promoters containing heat shock elements (HSE) to activate heat stress target gene transcription (Page et al. 2006) (Fig 3). In this study the expression of HSF1 was found to be greater than the expression of HSP70 in both seasons indicating the need of HSF1 trimers for the expression of HSP70 (Fig 2).

Expression of HSF1 and HSP70 under heat and cold stress:

In the present study, the expression of HSF1 and HSP70 increased with heat or cold stress. The expression of HSF1 and HSP70 increased from summer 1 (start of August) to summer 3 (mid September) and from winter 1 (mid November) to winter3 (start of January) (Fig 2). The expression of genes was significantly higher under acute stress i.e. in summer 3 and winter 3 (Fig 2). The HSF1 and HSP70 expression was elevated, from summer 1 by 0.43 and 9.46 folds; and 0.22 and 9.01 folds, respectively, in summer 2 and summer 3; and from winter 1 by 0.66 and 4.71 folds; and 1.71 and 3.83 folds, respectively, in winter 2 and winter 3. The expression of HSF1 was found to be greater than the expression of HSP70 in both seasons.

Table.1 Primer sequences used for SYBR Assay along with their efficiency

Gene	Accession No	Primers	Amplicon	Efficiency
HSP 70	EU 099315	Forward:5'-GGCTGGATGCCAACACCTT-3' Reverse:5-GCTCCAGCTCCTTCCTCTTGT-3'	64 bp	1.83
HSF 1	DQ 097507	Forward: 5'-TCCGGAGGTGGTCCACAT-3' Reverse: 5'-GAACTCGGTGTCATCCCTCTCT-3'	64 bp	1.85
18S	Universal	Forward: 5'- GGTTGATCCTGCCAGTAGCATAT-3' Reverse: 5'-TGAGCCATTCGCAGTTTCACT-3'	62bp	1.93
GMCSF	AY553190	Forward:5'- CTAGTCTCATGGGCTCCTTGAC-3' Reverse:5- TTTTGAAGGTGATAAACTGGGTTCCA-3'	102bp	1.89
IL-12	EF424254	Forward:5'- AAAAGCAGCAGAGGCTCCT-3' Reverse:5- GTCCACGCTGACCTTCTCT-3'	82bp	1.90
TNF-α	EF424255	Forward:5'-ACTCATATGCCAATGCCCTCATG -3' Reverse:5-GCAGGCACCACCAGCT -3'	68bp	1.93

Table.2 Correlation between HSF1, HSP 70 gene expression and milk yield in different seasons

Season	HSF1 and HSP 70	HSF70 and Milk yield	HSF1 and Milk Yield
Summer	0.99**	-0.207	-0.245
Winter	0.9445**	-0.187	-0.168
Overall	0.9770**	-0.151	-0.093

Figure.1a Real-time PCR amplification of HSP70 and HSF1

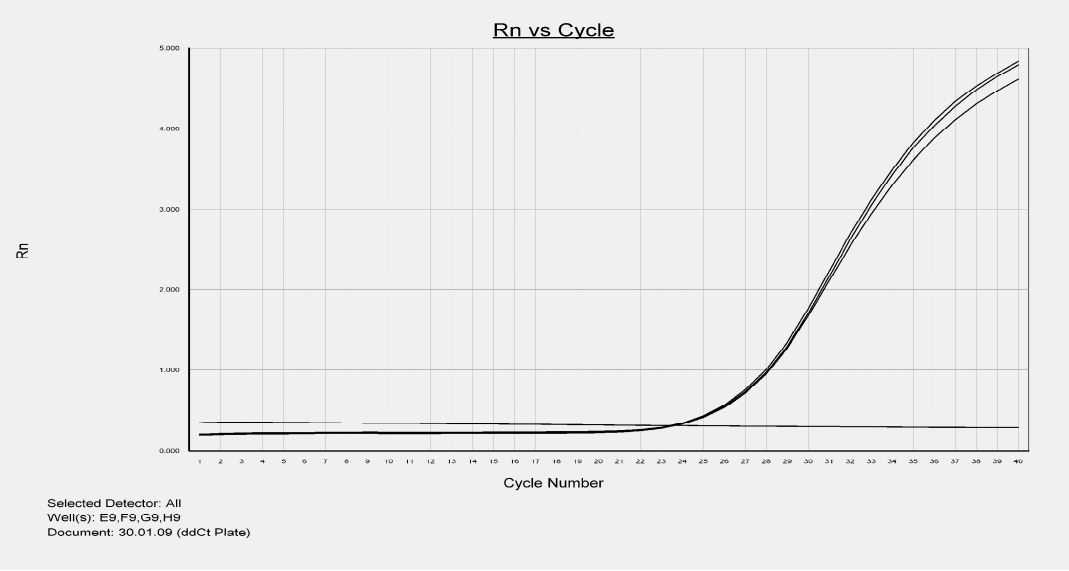
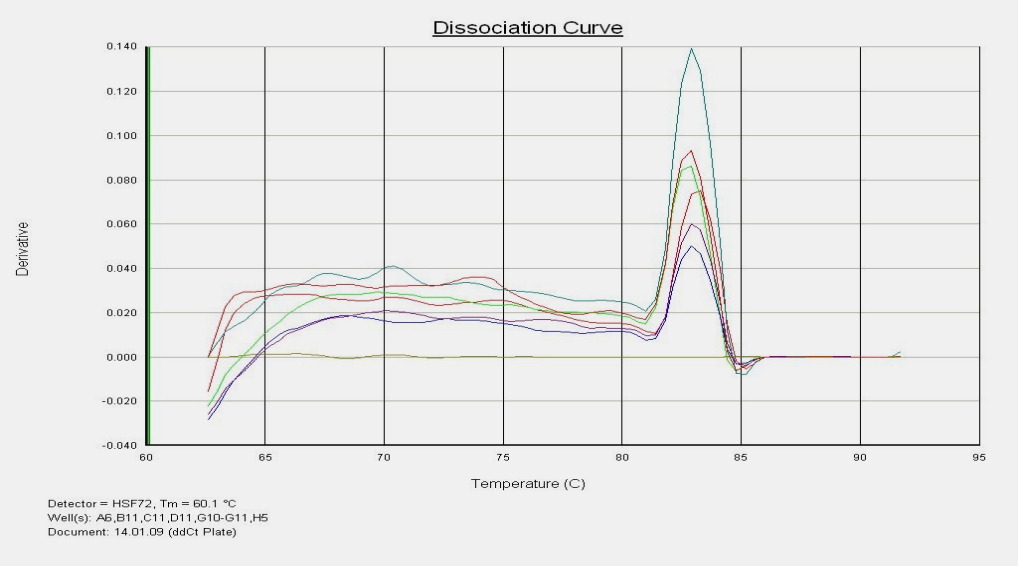


Figure.1b Melt curve analysis of SYBR Green real-time PCR product of HSP70 and HSF1 after 40 cycles



A significant positive correlation between the expression of both the genes (HSP70 and HSF1) in summer and winter (Table 2) indicated the need of the transcription factor HSF1 for the expression of HSP70.

Expression of HSP70, HSF1 and milk production

Milk production decreased with increase in the expression of HSF1 and HSP70 in animals under both heat and cold stress i.e. a negative correlation between milk production and both HSP70 and HSF1 expression in summer and winter was observed (Table 2). As the expression of both the genes increased to cope up with the stress there is a significant decrease in milk production because of the metabolic load.

Correlation between HSP70 and HSF1 with cytokines - IL-12, TNF-α and GMCSF

The expression of IL-12, TNF-α and GMCSF increased with increase in stress. The gene expression of IL-12, TNF-α and GMCSF was elevated, from summer 1 by 2.85 and 6.68 folds; 4.02 and 7.27 folds; and 4.66 and 4.71 folds, respectively, in summer 2 and summer 3; and from winter 1 by 2.09 and 5.05 folds; 4.05 and 5.81 folds; and 1.46 and 6.54 folds, respectively, in winter 2 and winter 3. The expression of HSP70 was positively correlated with the levels of IL-12 ($r = 0.909$; $P \leq 0.05$), TNF-α ($r = 0.794$) and GMCSF ($r = 0.662$). Similarly, the expression of HSF1 was also positively correlated with the levels of IL-12 ($r = 0.9186$; $P \leq 0.05$), TNF-α ($r = 0.7991$) and GMCSF ($r = 0.6793$). The three cytokines (IL-12, TNF-α and GMCSF) were also significantly ($P \leq 0.05$) positively correlated (IL-12 and TNF-α: $r = 0.9508$; IL-12 and GMSCF: $r = 0.8894$; TNF-α and GMCSF: $r = 0.8651$). HSP70 is termed to as a chaperokine to better describe the ability of HSP70 to act as a chaperone and cytokine (Asea et al ., 2000a ; Asea et al., 2000b; Asea et al., 2002).The interaction of HSPs with the antigen presenting cells leads to secretion of cytokines TNF-α, IL -1β, IL-12 and GM - CSF by macrophages (Srivastava, 2002).

Fig.2 Differential expression HSP70, HSF1 and cytokines (IL-12,TNF-α and GMCSF) in summer and winter. Levels not connected by same letter are significantly different ($P \leq 0.05$)

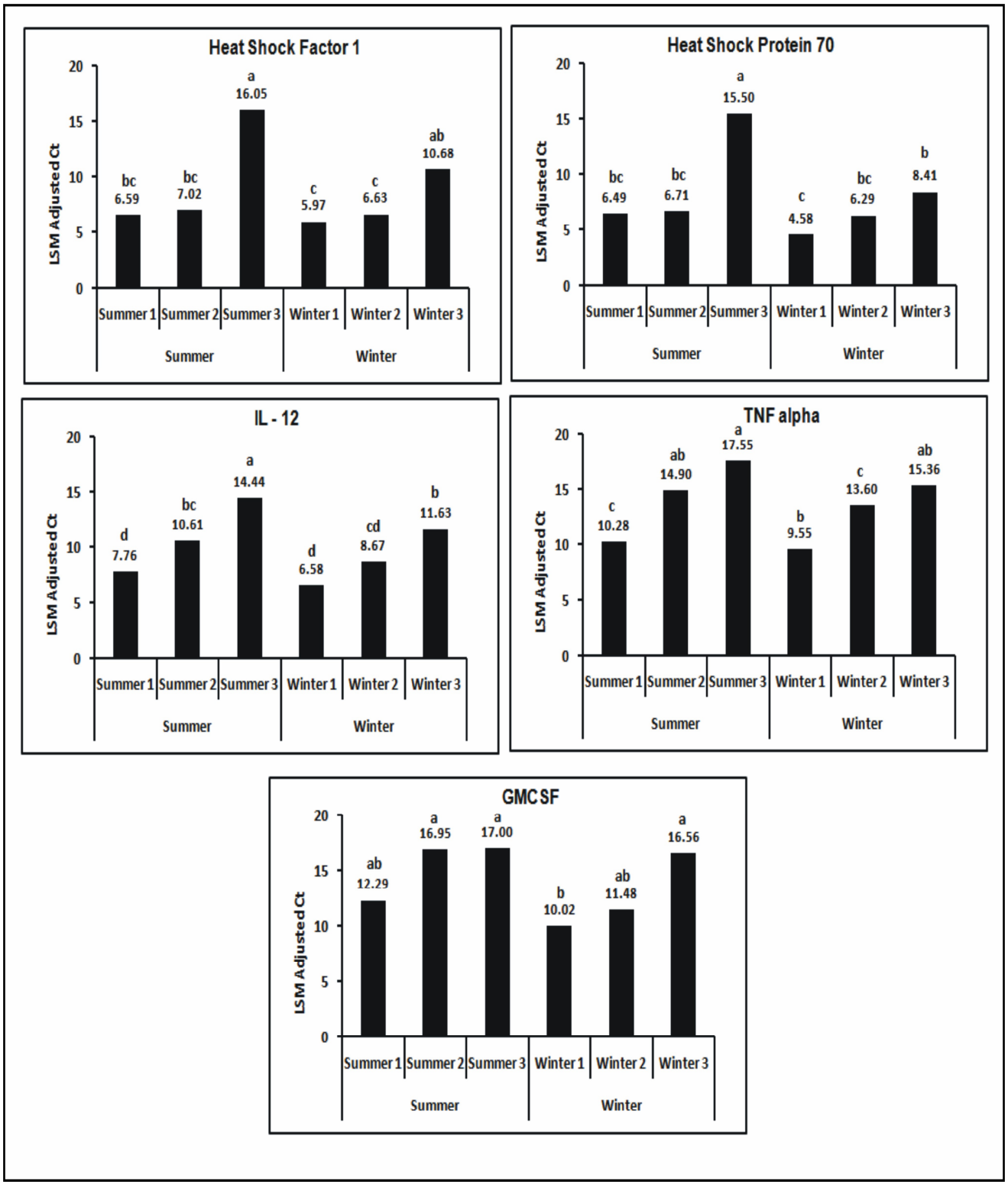
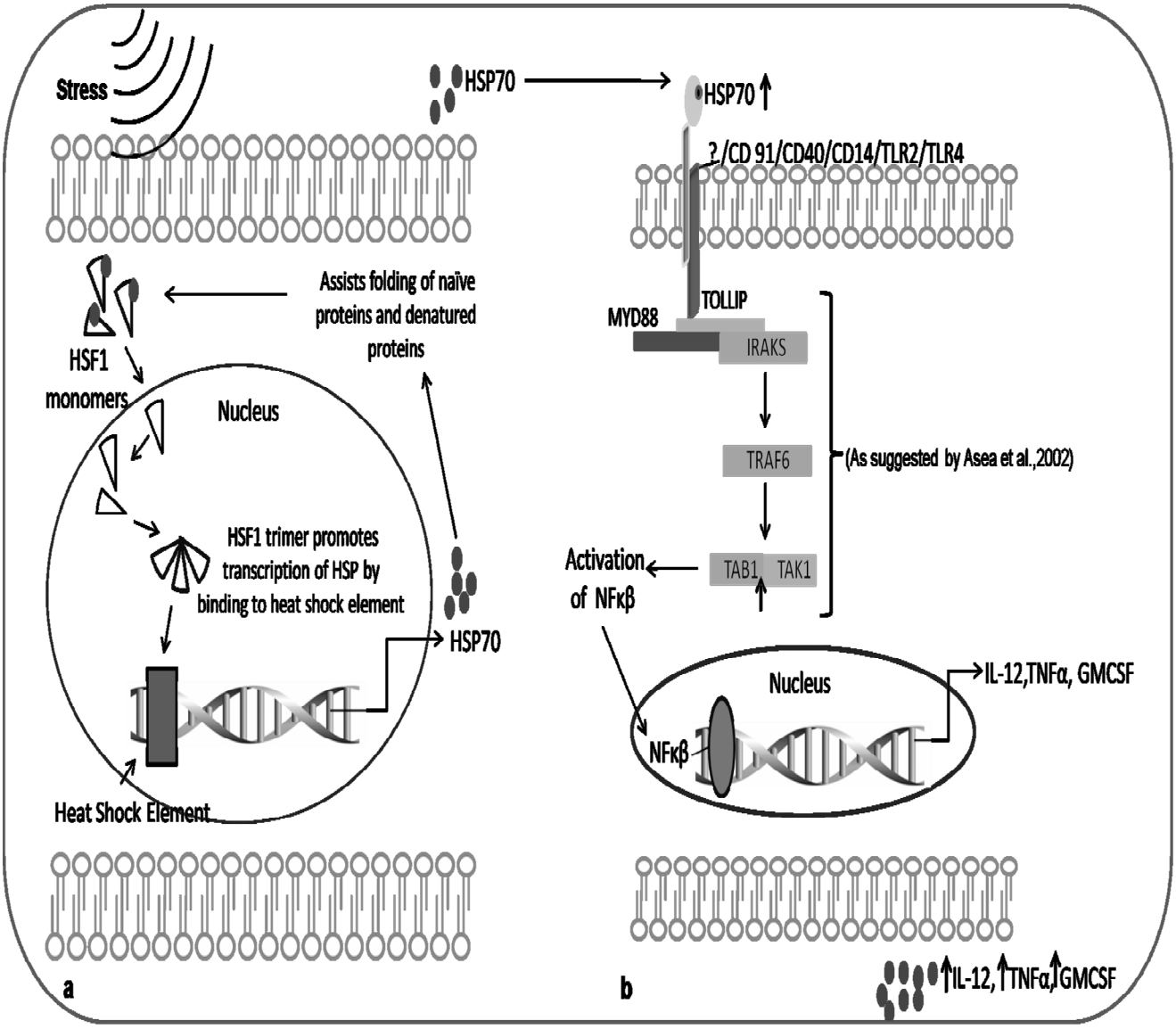


Fig.3a Hypothetical triggering of HSF1 and HSP70 under stress in the cell; Upon heat stimulus, the HSP dissociate from HSF1 monomers, which then unfold and bind to two other HSF1 monomers to form trimers before their nuclear translocation. Once in the nucleus, homotrimeric HSF1 binds promoters containing heat shock elements (HSE) to activate heat stress target gene transcription.



b.Hypothetical Signal transduction pathway explaining the correlation between Hsp70, IL-12, TNFα and GMCSF. Hsp70-induced cytokine production may be mediated via the MyD88/IRAK/NF-kappaB signal transduction pathway to transduce its proinflammatory signal under stress (Asea *et al*., 2002). The arrow marks indicate that in this study with increase of Hsp70 there was a proportional increase in the level of IL-12, TNFα and GMCSF.

Further, Hsp70 was proved possesses potent cytokine activity, with the ability to bind with high affinity to the plasma membrane, eliciting a rapid intracellular Ca^{2+} flux, activating NF-kappa β, and up-regulating the expression of cytokines in human monocytes (Asea *et al*. 2002). In this study it is observed that with the increase of HSP70 there was a proportionate increase in the level of TNF-α, IL-12 and GM-CSF indicates the representative role of HSP70 as a cytoprotectant under stress (Fig 3).

This study on differential expression of HSF1 and HSP70 is novel and first of its kind in buffaloes. To our knowledge no in-vivo comparative studies have been conducted in buffaloes to identify the differential expression of these genes under heat/cold stress. There was an increased expression of these genes under stress with a correlated increase in TNF-α, IL-12 and GMCSF.

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