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Seasonal variation in expression pattern of heat shock factor genes in *Ovis aries* and *Capra hircus*

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ABSTRACT

In many dairy animals the correlation between longevity and stress resistance has been observed, which suggests that, for the regulation of lifespan, the ability to sense and respond to environmental challenges is important. Therefore it is necessary to observe the role of heat shock factors (HSFs), in the regulation of longevity which acts as a master transcriptional regulator of stress-inducible gene expression and protein folding homeostasis. Exposure to heat stress causes changes which have a substantial impact on production and productivity. Therefore the four major mammalian *HSF* genes, *HSF-1, 2, 4,* and *5* have been studied in sheep and goat. Major objective of this study was to analyze the expression status of these genes in sheep and goat using gene-specific primers. Changes in the gene expression profile of these two species were noted by quantitative real-time PCR (qRT-PCR). The expression level in both the species has been studied and it was found that the level of *HSF-1, 2, 4* and *5* mRNA was higher in testis compared to all the tissues examined. Moreover, they are expressed in a wide range of tissues but their expression was variable. The analysis of seasonal changes in blood profile in goat and sheep showed an up-regulation in *HSF-4* and *HSF-5* genes in winter. The study implicates that the intricate balance of different *HSF*s is adjusted to minimize the effect of seasonal changes in environmental conditions. These findings enlighten our understanding of the complex, context-dependent regulation of *HSF* gene expression under normal and stressful conditions.

Key words: Goat, Heat shock factors, Seasonal changes, Sheep, Stress

In India, dairy animals occupy an important place in the agricultural economy because of their adaptability to harsh climatic conditions and their tolerance to tropical diseases and survival under poor feeding and management practices (Thiruvenkadan *et al.* 2013). Heat shock factors (HSFs) are recognized as the central component of the eukaryotic heat shock response. In higher eukaryotes, induction of heat shock genes is mediated by the activation of heat shock factor (HSF), which binds to a target sequence, the heat shock element (HSE), located in the promoters of heat-induced genes (Sorger 1991; Prasad *et al.* 2007; Naidu and Dinkova 2017). The activation of HSF involves its conversion from a cryptic non-DNA-binding form to a sequence-specific DNA-binding form in higher eukaryotes (Wu *et al.* 1990). High air humidity accompanied by high ambient temperature causes an additional discomfort and enhances the stress level which in turn results in depression of the physiological and metabolic activities of animals. Reactions of homeotherms to moderate climatic changes are compensatory and are directed at restoring thermal balance (West *et al.* 1999).

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The increasing concern of thermal discomfort for farm animals is debatable not only for countries of tropical zones, but also for nations of temperate zones in which ambient temperature is increasing due to climate change (Nardone *et al.* 2010). Animals undergo various kinds of stress, i.e. physical, nutritional, chemical, psychological and heat stress (Morimoto *et al.* 1994, Ahn and Thiele 2003). Among all, heat stress is the most concerning at present in the ever-changing climatic scenario. Heat stress results in decreased growth, reproduction, production, milk quantity and quality, as well as natural immunity, making animals more vulnerable to diseases, and even death. The increasing demand for animal products paralleled by the frequent hot climate is a serious threat for the agricultural sector (Sarangi 2018). Among the domestic ruminant species, goats are less susceptible to environmental stress as they possess water conservation capability, higher sweating rate, lower basal metabolism, higher respiration rate, higher skin temperature, and constant heart rate and cardiac output (Shkolnik 1972). Regardless of the breed, the breeding environment, and its climatic variables can trigger physiological, biochemical, hematological and hormonal alterations that result in a reduction in the heat production to maintain homeothermy (Attia 2016). Although, goats are very well adapted to hot and humid climatic conditions and considered as animals of greater hardiness than other ruminants but selection for

this tolerance has traditionally resulted in their impaired productive and reproductive performance (Sejian 2013). HSFs are widely recognized as functional participants in heat stress action. However, thus far, information on the members of the *HSF* gene family is not that much available in goat and sheep. There are few major HSFs characterized yet in vertebrates, viz. *HSF-1, 2, 4,* and *5* (Lal *et al.* 2014; Saju *et al.* 2018). The present study was directed to investigate the expression profile of *HSF- 1, 2, 4*, and *5* genes in distinct tissue in goat and sheep. We also demonstrated seasonal changes in the expression profile of these genes.

MATERIALS AND METHODS

Collection of tissue and blood samples: Samples of goat and sheep were collected in triplicates (brain, ovary, testis, liver, lung and blood) from the Gazipur slaughter house, New Delhi, India, with the help of an on-site veterinary officer. The tissue sample collected were stored in RNA later (Sigma-Aldrich) and for its storage in deep freeze (–80°C) for further use. Total 10 mL of peripheral whole blood samples of 5 similar breeds of goat and sheep were collected in sterile Vacutainer (Beckton-Dickinson, Franklin Lakes, NJ, USA) containing sodium heparin as an anticoagulant. The samples were then stored at 4°C and taken to the laboratory.

RNA isolation and cDNA preparation: Total RNA was isolated from 100 to 200 mg of all tissue samples using TRIzol reagent (Sigma-Aldrich) and treated with DNAse I (Fermentas) following manufacturer's instructions. The 2.4 µg of total mRNA was reverse transcribed with Superscript III cDNA synthesis kit (Invitrogen Canada Inc.) using hexamer primers according to manufacturers' instructions for all tissue samples.

Semi-quantitative Real-Time PCR (RT-PCR): Real time RT-PCR was performed to determine the basal expression level of *HSF-1, 2, 4* and *5* gene mRNA in different tissues of goat and sheep, using Light Cycler 480 II Real-Time PCR machine (Roche Diagnostics, USA). Primers were designed to span exon-exon boundaries to ensure cDNA specificity. Melting curve analysis, gel electrophoresis, and PCR product sequencing (Scigenome) were used to verify amplification of the correct target genes. Gene specific primers (Table 1) were designed to amplify the four *HSF* genes and one reference gene, *GAPDH*, were tested. Normalized ratios were determined for all runs using the delta delta-Ct method (ΔΔCt) (Livak and Schmittgen 2001). All qRT-PCR reactions were conducted on a Light Cycler 480 Real-Time PCR machine (Roche Diagnostics, USA). A final volume of each 10 µL PCR reaction containing - 5 µL of 2× SYBR Green PCR Master Mix (Thermo Scientific, USA), 500 ng of cDNA template and 0.25 µL each of forward and reverse primers (10 pmol/ml) was performed using following thermal cycling parameters 95°C for 10 min, followed by 40 cycles of 95°C for 15s, 60°C for 30s, 72°C for 30s. Each sample was in duplicate with three biological replicates. Analysis of real-time PCR (qRT-PCR) was performed by delta-delta-Ct method (Livak and Schmittgen 2001). The total RNA absorbed was normalized on the basis of the Ct value for the *GAPDH* gene. The variation of expression among samples was calculated by the $2^{-\Delta\Delta CT}$ method, with the mean delta Ct value for all the samples from control being used as a calibrator.

Statistical analysis: All the values are expressed as mean±standard error of mean (SEM). The statistical significance of differences in mRNA expressions of the *HSFs* was analyzed by one way ANOVA followed by Tukey's Multiple Comparison Test using SigmaStat software (Jandel Scientific Inc., CA, USA). Statistical significance was drawn at $P<0.05$ and $P<0.01$ levels.

RESULTS AND DISCUSSION

RNA integrity and purity: The integrity of total RNA was checked on 1.5% agarose gel using 1× TAE as electrophoresis buffer. Total RNA was in good yield in all the samples. The bands of 18sRNA and 28sRNA reflected the high quality of extracted total RNA. The purity and concentration of total RNA was checked using Nanodrop. The OD 260/OD 280 values of isolated RNA samples were more than 1.8 which showed that samples were free from the protein contamination. The concentrations of the RNA samples were ranging from 200–20,00 ng/µl.

Gene expression analysis and mRNA expression: Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as reference gene. Efficiency-corrected relative

Table 1. *HSF* and *GAPDH* gene primers with their sequences, product size, GenBank accession number, gene symbol, and annealing temperature

Primer name	Primer sequence (5′–3′)	Product size (bp)	GenBank accession number	Gene symbol	Annealing temperature
HSF-1RTF1	GCGCTCATCTGCTGGAGCCCG	144	NM_001076809.1	*HSF-1*	60°C
HSF-1RTR1	CTTCCGGAAGCCATACATGTTGAGCTG				
HSF-2RTF1	CCTCAGAAGATCCAGTGACCATGATGGA	168	NM_001083405.1	*HSF-2*	60°C
HSF-2RTR1	CAACCAGGAGATCTGGGTCTATGCTA				
HSF-4RTF1	ATCCGCTGGAGCCCGAGCGG	161	NM_001191202.1	*HSF-4*	60°C
HSF-4RTR1	CCACCCTGCTCGATGCTCACCAC				
HSF-5RTF1	CCAAGCCTACTATCCAACAGCTGTGCT	169	XM_003583530.1	*HSF-5*	60°C
HSF-5RTR1	CCAATTAGAAGGCAGAAATTCAACTGGATAGG				
GAPDHF	ACAGTCCATGCCATCACTGCC	266	NM_001289726.1	*GAPDH*	60°C
GAPDHR	GCCTGCTTCACCACCTTC TTG				

quantification of mRNA was obtained by Pfaffl method (2001). Here, the efficiencies of primer were determined by serial dilution of template cDNA sample and run in triplicate. The summer season values were used as calibrator. We selected blood and brain, since they are crucial in maintaining homeostasis when exposed to changes in environmental temperature. Liver has its role as the central metabolizing and detoxifying organ, and gonads (testis and ovary) because of their importance in reproduction. The qPCR assay was performed individually for all transcripts. The metabolism is reduced during heat stress and is controlled by hormones. The environmental temperature has a predominant effect on the activity of biochemical blood variables in comparison to other tissues in different physiological periods, hence taking blood as arbitrary constant, seasonal changes have been observed in both sheep and goat.

Expression pattern of HSF genes in goat: Analysis of expression of *HSF-1, 2, 4*, and *5* in different tissues such as liver, lung, ovary, testis and brain including blood in goat was performed (Supplementary Figs 1 and 2). The level of expression of *HSF-1, 2, 4* and *5* mRNA was found to be higher in testis compared to all the tissues in summer and winter both season ($P<0.01$) (Supplementary Figs 1 and 2). Previous study also showed the predominant of *HSFs* in testis and variable among other tissues, indicating specific regulations of their expression (Fiorenza *et al.* 1995; Lal *et al.* 2014). *HSF-1* level in ovary was significantly elevated compared to liver, lung and brain and significantly decreased compared to testis (Supplementary Fig. 1). *HSF-4* level in brain was significantly increased compared to liver, lung, ovary and significantly decreased compared to testis in summer season. In previous study, *HSF-1* and *2* was found to be upregulated in testis because of its significant role in spermatogenesis (Wang *et al.* 2004; Akerfelt *et al.* 2010). In contrast, apart from testis, *HSF-4* gene in brain showed the higher expression that correlates with its prominent role in the development and differentiation of sensory organs (Akerfelt *et al.* 2007). In winter, *HSF-1, 4,* and *5* levels were significantly elevated in ovary compared to liver, lung, brain and significantly decreased compared to testis. However, *HSF-2* level was found to be elevated in ovary and brain compared to liver and lung (Supplementary Fig. 2). Fiorenza *et al.* (1995) also investigated the patterns of expression of *HSF-1* and *HSF-2* in different tissues and found variations of the abundance of mRNAs for *HSF-1* and *HSF-2* in murine.

Expression pattern of HSF genes in sheep: Similarly in sheep, the expression of *HSF-1, 2, 4*, and *5* was analyzed in different tissues such as liver, lung, ovary, testis and brain including blood in summer and winter season. The overall expression profile of *HSF-1, 2, 4*, and *5* genes in different tissues of sheep studied in summer and winter season is shown in Supplementary Figs 3 and 4. The level of expression of *HSF-1, 2, 4*, and *5* genes were found to vary between most of the tissues examined. The expression level of *HSF-1, 2, 4* and *5* mRNA was found to be significantly higher in testis compared to all the tissues in summer and winter both season ($P<0.01$). In summer, *HSF-1* and *HSF-4* levels were found to be significantly increased in ovary compared to liver, lung and brain. Furthermore, *HSF-1* level was significantly elevated in brain compared to liver and lung and significantly decreased compared to ovary and testis (Supplementary Fig. 3). However, in winter *HSF-2* level was significantly decreased in ovary compared to all tissues ($P<0.01$) and in brain it is significantly decreased compared to testis and significantly increased compared to ovary. Although the *HSF-5* level was significantly decreased in ovary compared to testis and significantly increased compared to brain (Supplementary Fig. 4). A similar condition was also found for *HSF-1* and *HSF-2* gene expression in murine (Fiorenza *et al.* 1995). The expression data show different expression patterns of *HSFs* genes in different tissues. This different tissue specific expression of *HSFs* genes might arise from tissue specific factors of the heat shock regulatory network that regulates the heat shock response (Guisbert *et al.* 2013).

Seasonal comparison of expression profile of HSF genes in blood of goat and sheep: In this study to further

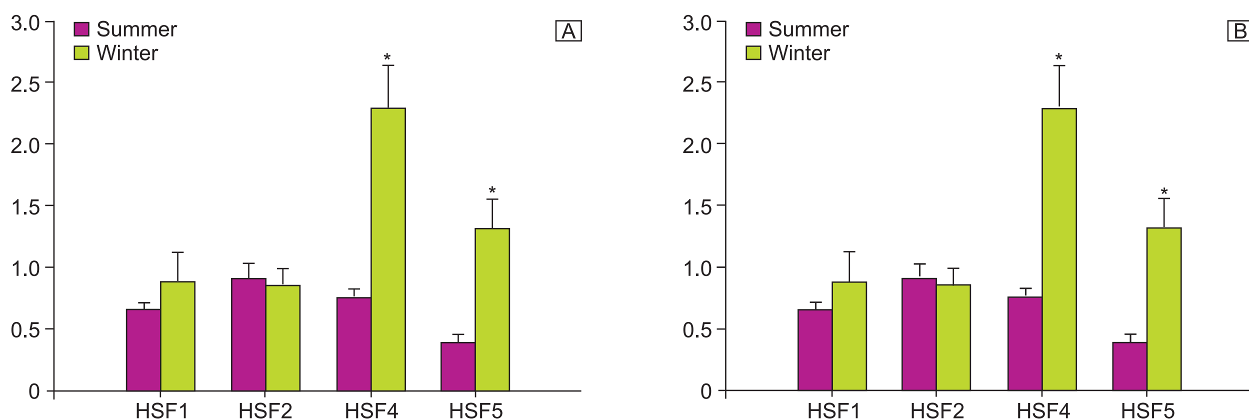

Fig. 1. Seasonal expression profile of *HSFs* genes in blood of goat (A) and sheep (B). RT-qPCR analysis of *HSF-1, 2, 4*, and *5* transcripts was performed in two different seasons. *GAPDH* was used as reference gene to normalize the *HSF-1, 2, 4* and *5* mRNA. Experiments were performed from blood collected from n=3 animals. Bar represent Mean±SEM. Statistical significance was drawn at $^*P< 0.05$.

characterize the expression of *HSF-1, 2, 4* and *5* genes in goat and sheep, we examined the expression of candidate genes in blood in two different seasons: winter (January), and summer (June), when daytime temperature of the environment and relative humidity in the north western region of the Indian subcontinent ranges from 7 to 19°C (80%), and 26–38°C (62%), respectively. The expression of *HSF-1, 2, 4* and *5* genes in peripheral whole blood samples of goat and sheep after an acclimatization period of 2–3 weeks in each season was determined, this is when the level of gene expression reached a stable stage to counteract the changes in the environmental conditions prevalent in that particular season. The seasonal changes in the expression profile of *HSF-1, 2, 4* and *5* genes are shown in Fig. 1. In the present study, a significant decrease in *HSF-4* and *HSF-5* levels was observed in summer season compared to winter season in goat and sheep ($P<0.01$). However, the *HSF-1* and *HSF-2* levels were unaltered. Our results are in agreement with previous reports where a significant decreased in *HSF-4* and *HSF-5* levels was observed in summer season compared to winter season in buffalo (Lal *et al.* 2014). Further, Lal *et al.* (2014) hypothesize that the reduced stability of these transcripts (*HSF-4* and *HSF-5*) in the summer may be due to physiologically higher body temperatures than normal.

Heat-responsive *HSFs* are ubiquitously expressed across various tissues that regulate responses to heat shock and other environmental stimuli. In addition to stress response, *HSF-1, 2, 4,* and *5* are also known to play important roles in development and gametogenesis. In conclusion, the level of expression of *HSF-1, 2, 4,* and *5* mRNA was found to be higher in testis compared to all the tissues in summer and winter both season, in goat and sheep. Moreover, they are expressed in a wide range of tissues but their expression was variable in all other tissues. The dissimilarity in the heat shock response among different tissues could support the hypothesis that the thermal limits of an organism/ individual may be governed by certain tissues more than by others. The study implicates that the intricate balance of different *HSFs* is adjusted to minimize the effect of seasonal changes in environmental conditions.

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