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Heat Shock Protein70 (HSP70) Gene Expression Pattern in Peripheral Blood Mononuclear Cells (PBMCs) during different Seasons in Sahiwal Cows (*Bos Indicus*)

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ABSTRACT

Thermal stress alters the normal body homeostasis and causes severe detrimental effects on production and productivity of animals. Heat shock proteins (HSPs) are highly conserved family of proteins that are ubiquitously expressed in animal's body during thermal stress. Out of all the members present in this family, HSP70 is regarded as the most significant indicator of thermal stress. The present study was therefore conducted to demonstrate the relative mRNA expression pattern of HSP70 gene in peripheral blood mononuclear cells (PBMCs) during different seasons in Sahiwal cows (Bos Indicus). Apparently healthy, non - lactating and non - pregnant sahiwal cows (above two years of age) were taken for study (n = 6). Blood samples were collected thrice i.e. once in December-January (THI <72), between February - March (THI = 72) and June (THI > 72). Blood sample collected in thermo - neutral zone (THI = 72) was taken as control. Quantitative real-time PCR (qPCR) study was undertaken to investigate the variation in relative mRNA expression profile of HSP70 gene during different seasons. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. The relative expression values of HSP70 during summer season were found statistically significant in comparison to winter. These results suggest that HSP70 gene expression varies with THI and this variation may play an imperative role in conferring thermo tolerance against heat stress during different seasons of a year.

Keywords: Thermal stress, Sahiwal cows, PBMCs, HSP70

Variations in ambient temperature either above the upper critical temperature or below the lower critical temperature leads to a condition known as thermal stress in most domestic animals including human beings. In tropical countries like India, heat stress due to high ambient temperature and humidity elicits deleterious effect on growth, production and reproduction of farm animals (Marai *et al*., 1995; Pandey *et al*., 2014). Heat stress occurs in animals when there is an imbalance between heat production within the body and its dissipation (Kumar *et al*., 2011). Temperature–humidity index (THI), a parameter that is extensively used to describe heat load on humans, is a good indicator of stressful thermal climatic conditions (McDowell *et al*., 1976). Heat stress causes a decline in milk secretion in dairy animals (Silanikove *et al*., 2009). Thermal stress is caused by an amalgamation of environmental factors *viz* temperature, relative humidity, solar radiation, air movement, and precipitation (Bohmanova *et al*., 2006). Cattle evolved in hot climatic conditions acquired genes that confer protection to the cells from detrimental effects of elevated temperature (Hansen, 2004). HSP70 is known to be a highly inducible chaperon and plays a key role to stabilize the native conformation of proteins and maintenance of cell survivability during thermal stress (Beckham *et al*., 2004). HSP70 is most responsive gene and can be used as a marker for determination of degree of heat stress (Kishore *et al*., 2013). HSPs account for 1%-2% of total protein in unstressed cell, however percentage

Table 1. Gene transcripts, primer sequence and resulting fragment size.

Target gene	Primer sequence	Predicted Size (bp)	References
HSP-70	For: 5'-GACGACGGCATCTTCAAG-3' Rev: 5'-GTTCTGGCTGATGTCCTTC-3'	132	Dangi *et al*., 2012
GAPDH	For: 5'-GCGATACTCACTCTTCTACTTTCGA-3' Rev: 5'-TCGTACCAGGAAATGAGCTTGAC-3'	82	U85042.1

is increased upto 4%-6% of cellular proteins when cells are heated (Crevel *et al*., 2001).

Many attempts have been made in past to investigate expression pattern of HSPs under thermal stress conditions in several species (Singh *et al*., 2014; Rajoriya *et al*., 2014; Dangi *et al*., 2012; Sharma *et al*., 2013), however few studies were aimed to evaluate the expression profile in indigenous breeds of cattle. The present *in vivo* study therefore was aimed to take an insight into mRNA expression profile of HSP70 during different seasons in PBMCs of Sahiwal cows.

MATERIALS AND METHODS

Reagents and media

Different chemicals *viz* chemicals for molecular biology and chemicals like trizol, ethanol, chloroform, DEPC, isopropyl alcohol, agarose, lymphocyte separation media (LSM) were procured from standard manufacturing firms like Sisco Research Laboratory (SRL), Himedia Qiagen and Fermentas.

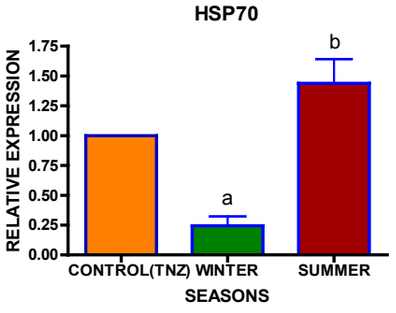

Figure 1. Relative mRNA expression pattern of HSP70 mRNA in PBMCs of Sahiwal cow (n=6 in each group). Data represents the mean ± SEM. Values bearing different superscripts differ significantly.

Geographical location

Present investigation was undertaken at instructional dairy farm, nagla of G.B. Pant University of Agriculture and Technology, Pantnagar. The place is located between 28° 53' 24'' to 30° 27' 50'' N and 77° 34' 27'' to 81° 02' 22'' E at 243.84 m MSL in Tarai region of Uttrakhand state.

Sample collection

Six non-lactating, non-pregnant and apparently healthy Sahiwal cows above two years of age were selected for the study. These animals were kept under similar managemental and nutritional regimen throughout the period of study. Six blood samples (5-7 ml) were collected aseptically by jugular vein puncture during peak winter (temperature range 3°C to 16°C, THI<72), thermoneutral (temperature range 16°C to 22°C, THI=72) and peak summer season (temperature range 30°C to 41°C, THI > 72). The thermoneutral season values were used as control. THI was calculated as per THI = 0.72(W°C+D°C) + 40.6 (Kadzere, *et al*., 2002), where W°C and D°C are wet and dry bulb temperature. Safety measures were taken to minimize the effect of ribonuclease during processing. All samples were processed within one hour of collection.

PBMCs isolation

Dilution of whole blood was done in phosphate buffer saline (PBS, pH=7.4) in the ratio of 1:1. PBMCs were isolated by using HiSepTM Lymphocyte separation media 1077 (LSM) (Himedia). Briefly, LSM was aseptically transferred to a 15 ml clean centrifuge tube and overlaid with diluted blood to produce a clean interface between the two layers. The mixture was centrifuged at 1500 rpm for 30 min. at room temperature. PBMCs fraction from the interface was collected gently. Further centrifugation was done for washing the cells with phosphate buffer saline

(PBS) (pH 7.4). Red blood cells lysis buffer was added to PBMCs pallet, mixed well and centrifuged. Supernatant was discarded and washing was repeated twice. Finally PBMCs pellet was re-suspended and transferred to a sterile (Diethylpyrocarbonate treated) micro-centrifuge tube.

Total RNA extraction and quality determination

Total RNA was isolated using Trizol reagent (SRL). Briefly, trizol reagent and chloroform were added to PBMCs pellet and mixed gently followed by centrifugation at 12,000 rpm for 15 min at 4°C. The initial upper aqueous layer was aliquoted into sterile eppendorff tubes and equal volume of ice cold isopropanol was added, vortexed gently followed by centrifugation at 12,000 rpm for 12 min at 4°C. Pellet was washed twice with 75% ethanol by centrifugation at 7500 rpm for 5 min at 4°C. The isolated total RNA was stored in nuclease free water (NFW) (Qiagen, India). Purity and concentration of total RNA was confirmed by using Nanodrop spectrophotometer (Thermo scientific, USA). Absorbance at 260 and 280 nm wavelengths was measured against NFW as blank. RNA samples showing the OD 260: OD 280 values between 1.8-2.0 were expected to contain no protein and were taken for further use. The integrity of total RNA was also checked using denaturing agarose gel (1.0%) electrophoresis and further visualized under ultraviolet light. Two bands (28S and 18S RNA) indicated the high quality of total RNA.

Reverse Transcription and Quantitative Real-Time PCR

Constant amount of 1 µg of total RNA were reversed transcribed to complementary DNA (cDNA) using cDNA synthesis kit (Fermentas) according to manufacture instruction. First strand cDNA was confirmed by amplification of GAPDH gene. Quantitative Real-time PCR (qPCR) was performed with SsoFast TM Eva Green ®Supermix kit (Bio-Rad, USA). A master mix of following components was prepared- NFW, forward primer, reverse primer (0.5 µM each) and SsoFast TM Eva Green ®Supermix. The master mix was added to strip tubes and cDNA template was added. The qPCR conditions were as follows, initial denaturation at 95°C for 30 s, denaturation at 95°C for 10 s, annealing at 58°C for 10 s and extension at 72°C for 15 s for 35 cycles and last cycle at 95°C for 60 s. No template control (NTC) was placed for gene quantification for checking the contamination in the reaction components other than the cDNA. After the run has ended, cycle threshold (C_t) values and amplification plot for all determined factors were acquired by using the "Eva green (with dissociation curve)" method of the real time machine (MxPro3005 Stratagene, Agilent technology, USA). The specificity of real time PCR products were checked by (1) analysis of melting temperature (Tm) of the product obtained from dissociation or melting curve (2) by 2% agarose gel electrophoresis to verify the exact amplicon size. Relative expression of PCR product was determined by the equation suggested by Pfaffl (2001). The primer sequences and expected polymerase chain reaction (PCR) product lengths are shown in Table 1.

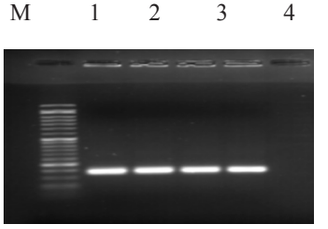

Figure 2. Confirmation of real time PCR products on 2% agarose gel electrophoresis. Lane M: 50 bp DNA ladder, Lane 1-4: HSP70 gene (132 bp), Lane 5: NTC

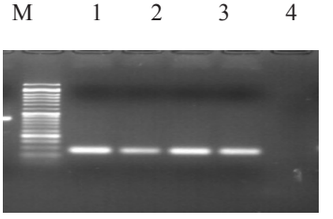

Figure 3. Confirmation of real time PCR products on 2% agarose gel electrophoresis. Lane M: 50 bp DNA ladder, Lane 1-4: GAPDH (82 bp), Lane 5: NTC

STATISTICAL ANALYSIS

The statistical significance of differences in mRNA expressions of examined factors was assayed by 'paired t test' with the help of SPSS 17.0 software. Differences were considered significant if $P < 0.05$.

RESULTS AND DISCUSSION

Stress is a broad term, generally used in negative connotation and is described as the cumulative detrimental

effect of a variety of factors on the health and performance of animals (Kumar *et al*., 2011). Rising environmental temperature coupled with humidity poses adverse impact on production and productivity of animals. Due to thermal stress a great proportion of energy is channelized to maintain normal body temperature and counterbalance the effect of heat stress (Mishra and Palai, 2014). Animals deal with these environmental stressors through behavioural and physiological means such as sweating, panting, drinking water, seeking shade, decrease in feed intake and by regulating their metabolic rates. Thermoneutral (Comfort) zone is a range of ambient temperature within which there is little or almost no fluctuation in body temperature with a negligible effort from thermoregulatory mechanism. A THI of either 72 or below is regarded as no heat stress, 73-77 as mild, 78-89 as moderate and over 90 as severe stress (Fuquay, 1981). At the cellular level, heat and other metabolic stressors induce the synthesis of a set of highly conserved HSPs (Lindquist and Craig, 1988). HSPs belong to the family of chaperones and play vital roles in regulating the proper folding of nascent proteins and refolding of denatured proteins (Lanneau *et al*., 2010). Studies have been conducted to investigate the effect of heat stress on HSPs expression under *in vitro* conditions (Kishore *et al*., 2013; Deb *et al*., 2014). However, very few studies have been carried to gain insight into the impact of thermal stress on expression profile of HSPs in indigenous cows under *in vivo* conditions. Therefore, the present investigation was undertaken to evaluate the impact of temperature and humidity on relative expression profile of HSP70 transcripts. In our study, the relative expression of HSP70 in Sahiwal during summer season was found significantly higher ($P<0.05$) than the corresponding values during winter season (Fig.1). Elevation in THI triggered substantial upregulation of HSP70 gene in Sahiwal cows. Our findings corroborate with the previous studies, where thermal stress induced rise in HSP70 expression in caprine PBMCs (Sharma *et al*., 2013; Dangi *et al*., 2012), leukocytes of buffalo (Pawar *et al*., 2014), PBMCs of cows (Lacetera *et al*., 2006), dermal fibroblasts of cattle (Singh *et al*., 2014), bull sperms (Rajoriya *et al*., 2014), PBMCs of pigs (Bambou *et al*., 2011), testis and epididymis of mice (Cao *et al*., 2009), mice liver (King *et al*., 2002), RBCs of rainbow trout (Currie and Tufts, 1997). Similar findings were also reported by Dangi *et al*. (2014) where rise in temperature dramatically elevated the expression of HSP70 promptly and a decline in temperature inhibited the over expression of HSP70 in a short period. Patir and Upadhyay (2010) indicated an elevation in the HSP70 concentration after 2 h of exposure at 45°C in Murrah buffaloes. Similarly, Zulkifli *et al*. (2010) observed that the transportation under hot, humid tropical conditions significantly augmented HSP70 densities in the renal cells of Boer does. Higher expression of HSPs during thermal stress suggests possible involvement of them to ameliorate detrimental effect of thermal stress so as to maintain cellular integrity and homeostasis (Dangi *et al*., 2012). The findings of present study suggest that expression of HSP70 is influenced by the THI of the season and its up-regulation during high THI may play a crucial role in providing defence against thermal injury at cellular level.

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