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"In-Vitro" Expression Profile of HSP 70.1 And HSP 90.1 Genes in Peripheral Blood Mononuclear Cells of Indian Native Tharparkar and Crossbred Vrindavani Cattle

Lakshya Veer Singh*, G Ravi Kumar, Shishir Kumar Gupta, Smita Gupta, Pragya Singh, Shweta Sharma, Aditya Prasad Sahoo1 and AK Tiwari*

Molecular Biology Laboratory, Division of Veterinary Biotechnology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

***Corresponding author:** AK Tiwari, Director, Molecular Biology Laboratory, Division of Veterinary Biotechnology, ICAR-Indian Veterinary Research Institute, India.

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Abstract

Circulating peripheral blood mononuclear cells (PBMCs) can be used as an effective model to understand the heat stress response of different cattle types and buffaloes. Environmental temperature is one of the significant abiotic influences that impact the normal biological function and productive performance of dairy cattle. This investigation was undertaken to determine the temporal profile of HSPs (HSP70.1 and HSP90.1) expression in circulating PBMCs isolated from Tharparkar and crossbred (Vrindavani) cattle in response to lethal heat shocks at T40 °C, T41 °C and T42 °C for 13 hours. The quantitative real-time reverse transcription (qRT-PCR) expression data showed significant increase in mRNA levels of the both HSPs genes in heat stressed PBMCs. Out of two HSPs, HSP 90.1 was relatively more expressed followed by HSP70.1 in these two cattle types, indicating its prominent role as molecular chaperone to stabilize the native conformation of proteins. Also, the response elicited was different for both the cattle as the level of expression of HSPs throughout the time period of heat stress was higher in crossbred cattle than in Tharparkar. Results indicated that PBMCs from crossbred Vrindavani cattle are less tolerant to heat exposure than those from Indian native Tharparkar cattle, which is associated with higher expression levels of HSP70.1 and HSP90.1 genes.

Keywords: PBMCs; Heat stress; HSP; Tharparkar; Crossbred; Vrindavani

Introduction

India has the largest livestock population in the world and ranks first in respect of cattle and buffalo population. Cattle constitute 37.5% of its total livestock population (BAHS Basic Animal Husbandry Statistics 2012) [1] and contribute around 50% of milk production in India (Rajoriya et al. 2014 and Sharma et al. 2013; Sengar et al. 2018) [2-4]. Indigenous breeds of cattle are well known for their thermotolerant capability and high disease resistance as well as major milk producing species in the Indian subcontinent. Indian zebu cattle (Bos indicus) breeds are well known for their adaptation to different agro-climatic conditions, their hardiness and survival under stressful conditions. As these cattle are thermotolerant, therefore, the these cattle have significantly less impact of heat stress on growth rate, milk yield as well as on reproductive functions (Hansen 2004; O'Bannon et al. 1955; Johnson 1965; Johnston et al. 1963; Chen and Qiu 2012) [5-9]. Among these cattle breeds, Indian native cattle breeds viz. Ongole, Sahiwal, Tharparkar etc. show much better heat tolerance than Bos Taurus and crossbred cattle (Rajoriya et al. 2014; Singh et al. 2014) [2,10].

Tharparkar is one of the most important dual-purpose breeds and are very resistant to many tropical diseases and have good heat tolerance ability (Rajoriya et al. 2014; Deb et al. 2013) [2,11]. During past few decades, due to crossbreeding program and mechanization of agriculture in the country, various indigenous cattle breeds which form the core of traditional agriculture are being diluted. Currently, there is around 22.1 million crossbred cattle population including Holstein Friesian (HF), Jersey crosses and Vrindavani in India (Narayan et al. 2007) [12]. Though the crossbred animals are known for high production, they are also found to be more susceptible to climatic stress conditions (Narayan et al. 2007; Deb et al. 2014; Driver and Khatib 2013) [12-14]

Heat shock proteins (HSPs) are the conserved protein and play an important role in protein folding and unfolding as molecular chaperones and in protection of cells from various stresses including heat stress (Mathew and Morimoto 1998; Li and Shrivastava 2004; Sodhi et al. 2013) [15-17]. HSPs are present in cells under normal conditions, but when cells are exposed to a higher temperature, the expressions of these proteins is up-regulated and play an important role in protein stabilisation and folding of denatured proteins in response to heat shock stress. Therefore, differential expression of HSPs under thermal stress may partially explain the relative stress tolerance of native breeds of cattle compared to exotic breeds (West 2003; Wheelock et al. 2010; Hu et al. 2016) [18-20]. In tropical, subtropical, and arid regions, high ambient temperature is one of the major factors affecting high producing animal production (Gill et al. 2017) [21]. Heat tolerance can be defined as "ability of the body to endure the impact of a hot environment without suffering ill-effects" (Bianca 1961) [22].

Heat shock proteins (HSPs) have been classified into five families (100, 90, 70, 60 and small HSPs) according to their molecular weight (Kristensen et al. 2004; Kumar et al. 2015; Verma et al. 2016) [23-25]. These are a group of highly conserved proteins which are expressed by all living organisms. HSPs are components of anti-stress mechanisms and allow cells to adapt to gradual environment changes. These molecular chaperones encompass several families in which HSP70 and 90 are the most temperature sensitive and highly conserved of the HSPs. HSP70 and HSP90 proteins protect cells from thermal or oxidative stress by temporarily binding to hydrophobic residues exposed by stress, these HSPs prevents these partially denatured proteins from aggregating, and allows them to refold (Kregel 2002; Picard 2002; Deb et al. 2014; Kumar et al. 2015) [26,27,13,24].

The objective of the present study was to characterise the stress response of PBMCs from Indigenous (Tharparkar) and crossbred cattle (Vrindavani) to heat challenges of varying temperatures and exposure times based on the expression profile of HSP70.1 and HSP90.1 genes.

Materials and Methods

Experimental animals

The present study was conducted on Tharparkar (Bos indicus) and Vrindavani crossbred cow, maintained at cattle and buffalo farm, Indian Veterinary Research Institute, Izatnagar. Vrindavani are crossbred cattle, which have recently been developed, and have the exotic inheritance of Holstein-Friesian, Brown Swiss, Jersey and indigenous inheritance of Hariana cattle (Singh et al. 2011) [28]. About 8-10 ml of venous blood was collected from each animal (15 animals of each breed) under sterile conditions from jugular vein in vacutainer containing EDTA.

Isolation of PBMCs

The PBMC were isolated by density gradient centrifugation (Singh et al. 2014). Briefly, blood was diluted, layered over Ficoll-Histopaque-1077 (Sigma, MO, USA), and centrifuged. The mononuclear cell band was recovered and washed twice in phosphate buffer saline (PBS). Residual red blood cells were eliminated by hypotonic shock treatment using redistilled water. The PBMC recovery and viability were determined cell counter

slide (Countess, Invitrogen, USA) and cell number was counted in a cell counter (Countess, Invitrogen, USA) count using the trypan blue exclusion method. Viability of PBMC typically exceeded 80% both in Tharparkar cattle and Vrindavani cattle. The PBMC were resuspended at 1×106 viable cells/mL in Roswell Park Memorial Institute 1640 medium (RPMI 1640 medium) (HiMedia, India) containing 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HiMedia, India) supplemented with 10% heat-inactivated fetal bovine serum, 100 U of penicillin, 100 µg of streptomycin, and 0.25 µg of amphotericin B/mL (HiMedia, India). The time between blood collections and establishment of cultures was less than 6 h.

Heat treatment

The PBMC isolated from the 15 Tharparkar and Vrindavani cattle were subjected to heat shock treatment. Cells were divided into 4 samples viz. control (T1), T2, T3, T4 each receiving different heat treatments. The 65-h incubation represents an optimal time to guarantee cell viability and elicit maximum proliferative response in bovine PBMC (Lacetera et al. 2006) [29]. Control cells were exposed to 37°C continuously for 65 h or while treatments cells were kept at 40, 41, or 42 °C, respectively, for 65 h. Treatment at 37 °C was adopted to mimic normothermia; 40, 41 and 42 °C groups mimicked conditions of severe hyperthermia.

PBMC cultures for Proliferation assay

The PBMC (100 µL) were added into quadruplicate wells of 24- well, flat-bottomed tissue culture plates and each samples put in four times. Tissue culture plates were subjected to the treatment protocol in an atmosphere of 95% air and 5% CO_2. After the first 24 h of incubation, cells were stimulated with Phytohaemagglutinin (PHA) (Sigma) of RPMI 1640 was added to each well. The mitogen PHA was added at final concentration of 5 µg/mL. Control wells contained 100 µL of PBMC suspension (1 × 106 viable cells/mL) without PHA (unstimulated). Cells were harvested at all times points of incubation period of 13 h.

PBMC cultures for HSP70.1 and HSP90.1 quantification

Intracellular mRNAs for HSP70.1 and HSP 90.1 were evaluated in PBMCs (500 µL of cell suspension containing 1×106 cells/well) cultured under the conditions described above.

Analysis of HSP70.1 and HSP90.1 mRNA levels:

Levels of HSP70.1 and HSP90.1 mRNAs were measured by qRT PCR. Total RNA was isolated from unstimulated and stimulated cells of each heat treatments using Tri reagent (Sigma-Aldrich) following the procedure described by Singh et al. (2014) [30]. The purity and concentration of RNA was checked by a NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific Inc. Wilmington, DE, USA). Prior to cDNA synthesis, RNA was treated with DNase treatment to remove any genomic DNA contamination. One µg of RNA was reverse transcribed using a RevertAid™ First Strand cDNA Synthesis Kit (M/s MBI Fermentas Life Sciences, Maryland, USA) according to the manufacturer's protocol.

The resulting complimentary DNAs (cDNAs) were used in quantitative real-time polymerase chain reaction (qRT-PCR). The qRT-PCR for HSPs and housekeeping genes (Beta-actin) was performed in triplicate (technical replicate) using HotStart-IT SYBR Green qPCR Master Mix (2X) (Affymetrix), and primers for HSP70.1, HSP90.1 and Beta actin were used. Primers were used previously reported by Dangi et al. 2012 [31]. The sequences of primers are as follows: bovine Hsp70.1 5'-GACGACGGCATCTTCAAG-3' (sense) and 5'-GTTCTGGCTGATGTCCTTC-3' (antisense); HSP90.1- 5'-GCATTCTCAGTTCATTGGCTATCC-3' (sense) 5'-GTCCTTCTTCTCTTCCTCCTCTTC-3' (antisense) bovine Beta actin 5'- CTACCTTCAATTCCATCATG -3'(sense) and 5'- GCGATGATCTTGATCTTC-3' (antisense) and Beta actin 5'- CTACCTTCAATTCCATCATG-3' (Sense) and 5'-GCGATGATCTTGATCTTC. The predicted sizes of PCR products are 132 bp for HSP70.1; 190 bp for HSP90.1 and 163 bp for Beta actin. The cDNA samples were amplified in a real-time fluorescence thermal cycler (Fast 7500 Real Time PCR system, Applied Biosystem, USA) in 96 well plate. Thermal cycling conditions were 95 °C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The relative expression of each sample was calculated using the 2−ΔΔCT method (Schmittgen and Livak 2008; Doley et al. 2014) [32,33]. Results were analyzed and shown as fold change (2−ΔΔCT) relative to the control group. All the reactions were performed in triplicate and the detected expression values werenormalized against the endogenous control.

Statistical analysis:

All Real-time PCR reactions were run in triplicates. The statistical analysis was done using JMP 9.0 (JMP®, Version 9.0. SAS Institute Inc. Cary, NC, 1989–2007). As all the comparisons made above are pairwise comparisons, t-test was done to test the statistical significance.

Results and Discussion

In the present study, PBMCs isolated from Indian Tharparkar and Crossbred cows (Vrindavani) were utilized as cellular in-vitro model to assess the comparative cellular tolerance during heat stress. The transcriptional induction of HSP70 and HSP90 mRNAs was used as indicators to evaluate the comparative cellular tolerance ability of both the cattle types.

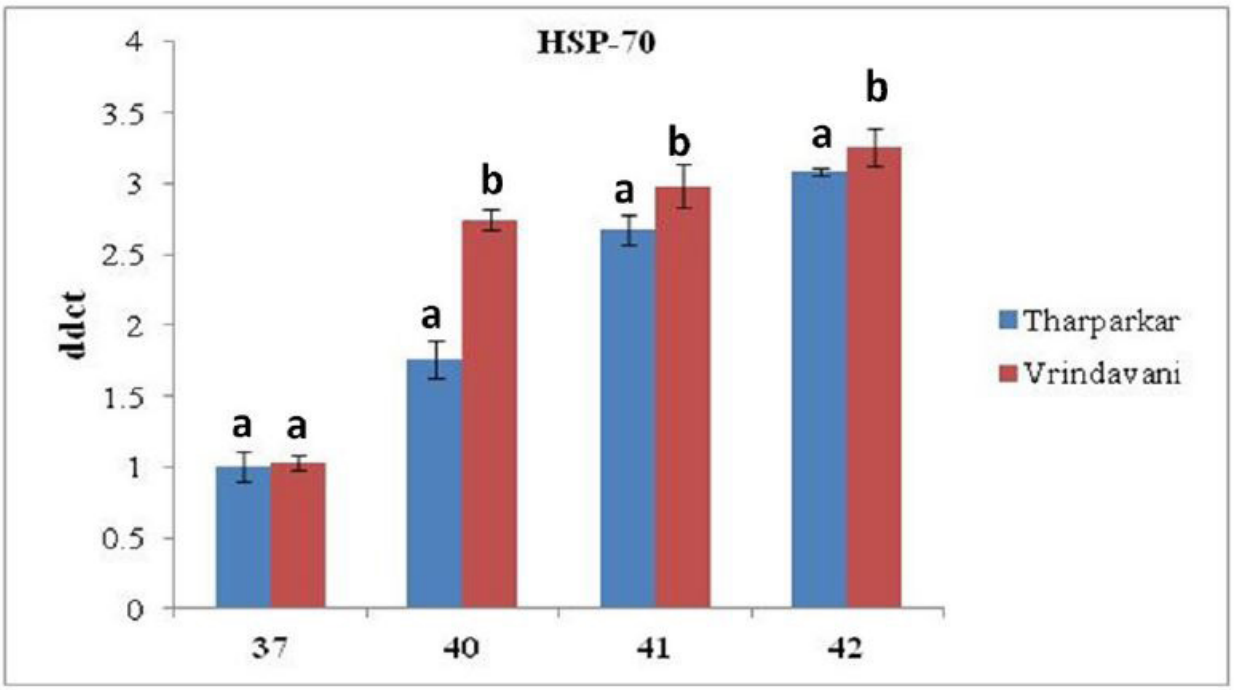
PMBCs were challenged with different high temperature at 37°C, 40°C, 41°C and 42°C for 65 h, respectively in two bovine species. The relative expression of HSP70.1 and HSP 90.1 increased significantly ($P < 0.05$) with increase in exposure temperature in both the cattle during the entire period of heat stress. Real-time PCR analysis revealed that the expression of HSP70.1 was upregulated by 1.00±0.10 at 37°C; 1.75±0.13 at 40°C ; 2.67±0.17 at 41°C and 3.08± 0.02 at 42°C and the expression of HSP90.1 by 1.11±0.08 at 37°C; 1.28±0.13; 1.40±0.16 at 41°C and 1.50± 0.09 at 42°C in Tharparkar cattle while in Vrindavani cattle, expression of HSP70.1 was up-regulated by 1.02±0.05 at 37°C; 2.74±0.07 at 40°C; 2.98±0.15 at 41°C and 3.25±0.13 at 42°C and HSP90.1 by 1.21±0.06 at 37°C; 1.41± 0.06 at 40°C; 2.11±0.09 at 41°C and 2.51±0.06 at 42°C. Increasing incubation temperature in both breeds increased the expression of both the HSPs genes, however, the expression of HSP70.1 and HSP90.1 were more pronounced in the PBMCs isolated from Vrindavani cattle than those from Tharparkar cattle (Figure 1).

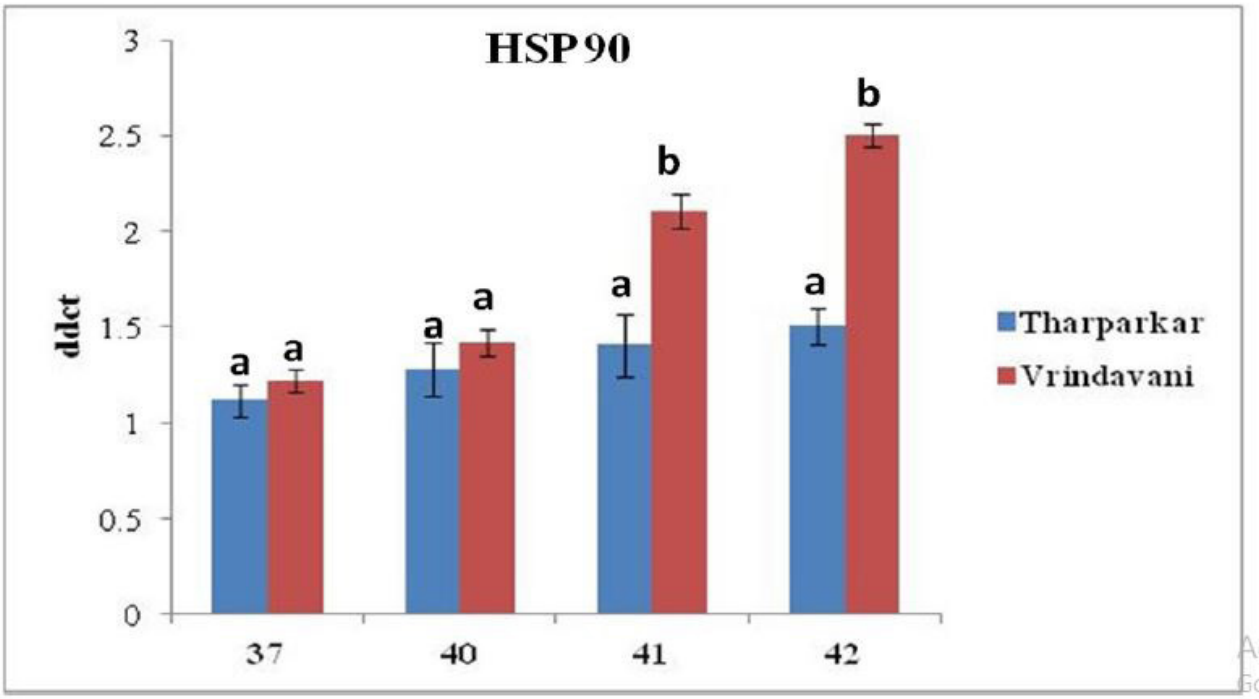
High environmentally friendly temperatures directly or indirectly affects the efficiency of dairy cattle. Similarly, heavy beef cattle are unable to resist thermal stress compared to lighter cattle because high fat unseating averts heavy cattle from adaptable their body temperature proficiently (Pragna, et al. 2017).

A number of previous studies performed in bovine or other species estimated mRNA levels of HSP genes following exposure to heat stress. In our study, we have found that though the expression levels of both the HSP mRNAs were up-regulated in response to heat stress, it was more pronounced in PMBCs isolated from crossbred Vrindavani cow. PBMCs from Tharparkar cow expressed lower amount of both the HSPs at all temperatures. This pattern of heat stress response suggests that Indian native cattle breed Tharparkar is more thermotolerant than the crossbred cattle Vrindavani. Lower amount of HSP may results from lower protein denaturation and thus, better resistance to heat stress. Previous studies have reported the similar findings. A lower amount of HSP70 was detected in the two thermotolerant breeds (Brahman and Senepol cattle) when the isolated PMBCs were subjected to heat stress (Kamwanja et al. 1994; Mehla et al. 2014) [34,35]. Another study investigated it further and reported that reduced HSP70 expression in heat stressed Brahman and Senepol cattle might be indicative of reduced protein denaturation as denatured proteins serve as signal for HSP70 synthesis. Lacetera et al. (2006) [29] also observed that when PBMCs isolated from the Holstein cows given a chronic heat shock, produced less amount of HSP72 than those isolated from the Brown Swiss cows and predicted the lower tolerance of Brown Swiss cows was associated with higher expression of HSP72.

The expression profile of different HSP transcripts was measured in PBMCs during heat shock condition. The reason for selecting four major HSP genes (HSP70 and HSP90) 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. It is also important that though heat shock response is an evolutionary conserved mechanism, different breeds/species may vary in their inherent ability to induce HSP synthesis during heat shock

However, these are several reports where high expression of HSP was co-related with increased thermotolerance (Angelidis et al. 1991; Horowitz 2002; Hansen 1999; Horowitz 2002; Meza-Herrera et al. 2006; Maibam et al. 2017) [36-39]. It has been observed that animals well adapted to warm climates produce lesser amounts of HSP proteins (Kamwanja et al. 1994; Lacetera et al. 2006; Romero et al. 2013) [34,29,40]. The activation of HSP genes is also related to the defense against cell damage from heat shock (Schiaffonati and Tiberio 1997; Rajhans et al. 2010) [41-48] and up-regulation of HSP72 expression might function as an indicator of cellular stress levels (Kristensen et al. 2004) [23].





A. Assessment of relative mRNA expression level of HSP 70.1 gene in heat stressed PBMCs isolated from Tharparkar and Vrindavani cattle at 37°C, 40°C, 41°C and 42 °C respectively. Data are represented as mean ± s.e.m. Levels (within time) not connected by same letter are significantly different (P<0.05).

B. Assessment of relative mRNA expression level of HSP 90.1 gene in heat stressed PBMCs isolated from Tharparkar and Vrindavani cattle at 37°C, 40°C, 41°C and 42 °C respectively. Data are represented as mean ± s.e.m. Levels (within time) not connected by same letter are significantly different (P<0.05).

Conclusion

In conclusion, our study found that bovine PBMCs in response to heat stress produced HSPs, but the expression levels of HSPs were different in both the cattle breeds and showed difference in their ability to ameliorate the deleterious effect of heat stress so as to maintain cellular integrity. PBMCs isolated from crossbred cattle produced higher expression levels of HSP70 as well as HSP90 than those isolated from heat-adapted Tharparkar cattle. Although heat shock response is an evolutionary conserved mechanism, different breeds/species as seen in the present study may vary in their inherent ability to induce HSP synthesis during heat shock. We suggest that increased thermotolerance of Tharparkar cattle is associated with the reduced production of HSPs, as expression of these stress proteins reflects extent of cellular damage.

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