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Differential effect of thermal stress on HSP70 expression, nitric oxide production and cell proliferation among native and crossbred dairy cattle

V. Bhanuprakash [a,*], Umesh Singh [a], Gyanendra Sengar [a], Basavaraj Sajjanar [b], Bharat Bhusan [c], T.V. Raja [a], Rani Alex [a], Sushil Kumar [a], Rani Singh [a], Ashish Kumar [a], R.R. Alyethodi [a], Suresh Kumar [d], Rajib Deb [a,*]

[a] Molecular Genetics Laboratory, ICAR-Central Institute for Research on Cattle, Meerut 250001, Uttar Pradesh, India
[b] School of Abiotic Stress Management, ICAR-National Institute of Abiotic Stress Management, Baramati, India
[c] Division of Animal Genetics and Breeding, ICAR-Indian Veterinary Research Institute, Izatnagar 243122, Uttar Pradesh, India
[d] Quality Control Laboratory, ICAR-Central Institute for Research on Cattle, Meerut-250 001, Uttar Pradesh, India

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ABSTRACT

In a tropical country like India, thermal stress is one of the major factors which significantly affects the productivity of dairy cattle. The present study was aimed to identify the effect of heat and cold stress on cell viability, mitogen stimulation indices, nitric oxide production and HSP70 expression in Sahiwal and Holstein crossbred (Frieswal) population in India. The results indicated that the Sahiwal breed can better withstand the effect of heat and cold stress significantly ($P < 0.05$) when compared to the crossbred cattle due to the higher survivability of the Peripheral Blood Mononuclear Cells (PBMCs) and Phytohemagglutinin (PHA-P) mitogen based stimulation indices. The study also revealed the significant differences ($P < 0.05$) in the level of nitric oxide (μM) production amongst the pre and post thermal stressed samples of Sahiwal and Frieswal crossbred samples.

Further, the expression of HSP70 was significantly ($P < 0.05$) higher in Sahiwal compared to Frieswal immediately after heat/cold shock to 6 h of recovery as indirect ELISA analysis showed gradual rise in the Hsp70 protein concentration (ng/ml) immediately after heat and cold stress (0 h) and reached the peak at 6 h of recovery. Western blot and immune fluorescent assay results were also corroborated with the findings of indirect ELISA. In Sahiwal cattle the mRNA expression of HSP70 and its protein concentration were higher ($P < 0.05$) during peak summer (44 °C) and winter (10 °C) as compared to Frieswal cattle. This investigation supports the earlier information on the higher adaptability of indigenous cattle breeds to hot and humid conditions compared to the crossbreds of temperate cattle breeds.

1. Introduction

Climate change is one of the major threats to the livestock industry in India, where the temperature in different seasons varies from 10 °C to 44 °C. High environmental temperature together with other extreme weather components like intense solar radiation and humidity exerts a negative influence on the performance of dairy cattle and increases their susceptibility to the diseases. Ingram and Mount (1975) observed significant reduction in the feed intake, growth efficiency and reproductive ability among the animals exposed to heat stress. Like homeotherms, bovine regulate internal body temperature by matching the amount of heat produced through metabolism by dissipating the heat from the animal to the surrounding environment. Different cellular mechanisms to alleviate the thermal stress are proposed and explained in animals. One of the prominent cellular responses under heat stress include transcriptional activation and accumulation of heat shock proteins (De Maio, 1999).

Among different heat shock protein (HSP) families, HSP70 is believed to be critically involved in heat stress response of cells. Since its accidental discovery in thermally stressed drosophila by Ritossa (1962), HSP70 is considered to play an important

* Corresponding authors.
E-mail addresses: bhanupvhn24@gmail.com (V. Bhanuprakash), drrajibdeb@gmail.com (R. Deb).

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role in cellular protection against many acute environmental or physiological stimuli (Morimoto et al., 1994; Maloyan et al., 1999). HSP70 family is related to the acquisition of thermotolerance and the more rapid recovery of heat-induced denatured proteins in their native state (Maloyan et al., 1999). Hsp70 is also involved in the disposal of damaged or defective proteins by ubiquitination and proteolysis pathways (Luders et al., 2000). In addition to improving the overall protein integrity, Hsp70 directly inhibits apoptosis (Beere et al., 2000). Some members of the Hsp70 family are expressed constitutively, and others are strictly stress inducible which are upregulated in response to hyperthermia, oxidative stress, and changes in pH (Milarski et al., 1989). The present study was designed to investigate the effect of heat and cold stress on cellular physiology and expression profile of bovine HSP70 on native and Holstein-crossbreed cattle maintained in India.

2. Materials and methods

2.1. Experiment I

2.1.1. Experimental animals and sample collection

Twelve clinically healthy, non lactating adult cows (six each from Sahiwal and Frieswal cattle) were randomly selected from Military Farm, Meerut, Uttar Pradesh, India. Blood samples were collected by jugular venipuncture in heparin coated BD vacutainer tubes. Immediately after collection, blood samples were stored at 4 °C in a portable refrigerator and transported to the laboratory for PBMC isolation.

2.1.2. PBMC cell preparation and cell viability

PBMCs were separated by Ficoll density gradient centrifugation using Ficoll-Paque Plus solution (APB, Milano, Italy) as per the manufacturer's recommendation. Fresh blood samples were diluted in 1:2 ratio using Alsever's solution, layered carefully over an equal volume of Ficoll-Paque Plus solution (APB, Milano, Italy) without intermixing and centrifuged at 400*g* for 40 min at 20 °C. The mononuclear cell band was recovered and washed twice in phosphate-buffered saline (PBS, Sigma, Milano, Italy). The efficiency of PBMC recovery and viability were determined by the trypan blue exclusion method using hemocytometer.

2.1.3. Grouping of PBMC cells and in vitro culturing

Isolated PBMCs from both the breeds were divided into three parts, one for exposing to heat stress, second for cold stress and the third as negative control where no stress was applied. PBMC cells were resuspended @1×10^6 viable cells/ml in RPMI 1640 medium containing 25 mM HEPES + 10% heat inactivated FBS, 2 mM L-glutamine, 100 U of penicillin, 100 µg of streptomycin and 0.25 µg of amphotericin B/ml (Sigma-Aldrich, USA) in 24-well plates. The time gap between blood collection and establishment of cultures was less than 3 h.

2.1.4. Heat and cold shock treatment

The basal level of cell viability before heat and cold treatments was evaluated before plating of the cells. The cells were stored at −80 °C until mRNA isolation and protein expression analysis of HSP70. The cells were given heat and cold treatment by keeping the cultured cells at 42 °C in a water bath and at 4 °C in refrigerator, respectively for one hour. Cells were plated @1×10^6 viable cells/ml in 24-well plates and incubated at 37 °C in a 5% CO_2 incubator for 8 h (recovery time). The cell viability and kinetics of HSP70 gene expression and synthesis were studied at 0, 2, 4, 6 and 8 h of recovery. At each recovery time, a fraction of cells was evaluated for cell viability and the other fraction was stored at −80 °C for HSP70 transcript and protein studies.

2.1.5. Lymphocyte proliferation assay

Lymphocyte proliferation test was used for the determination of a specific cellular sensitization. The lymphocytes were suspended @1×10^6 viable cells/ml in RPMI 1640 with previously described additives. 200 µL of diluted cell suspension per well was placed in a 96 well flat bottomed tissue culture plate in triplicate. Phytohemagglutinin (PHA-P) was used as a mitogen at the concentration of 5 µg/ml as suggested by Soper et al. (1978). The cells from different groups (Control, heat stress and cold stress) were allowed to proliferate with and without PHA-P. Cultures were incubated at 37 °C, in a humidified CO_2 incubator with 95% air and 5% CO_2 for 24 h. The proliferative response of lymphocyte was determined by using the colorimetric MTT (tetrazolium) according to the protocol developed by Mosmann (1983). The lymphocyte blastogenic response was expressed as a stimulation index (SI) and was calculated as the ratio of the ODs of the stimulated and unstimulated cells at 530 nm. Data from different experiments were presented as mean ± SE.

2.1.6. Nitric oxide estimation

The RPMI 1640 complete medium supplemented with 5 mM of L-arginine was used for nitric oxide estimation assay. 100 µL of the cell suspension containing 1×10^6 PBMC from each group were plated in triplicate in 96 well plates. Geranyl geranyl acetone (GGA) – an inducer of HSP70 (Sigma, USA) and LPS (positive control) were added at the final concentration of 40 and 10 µg/ml, respectively. RPMI was used as the negative control. The plates were incubated at 37 °C in a humidified CO_2 (5%) incubator for 24 h. Supernatants were collected from all the wells and stored at −20 °C until nitric oxide (NO) estimation. For NO estimation, $NaNO_2$ (sodium nitrite) in different concentrations was used as standard. In a 96-well ELISA plate, 50 µL of the cell culture supernatant or 60 µL of Griess reagent (Sigma Aldrich, USA) as standard was added. The plates were incubated at 37 °C for 30 min A_{550} reading was taken by a microplate ELISA reader. By using the standard curve ($NaNO_2$ concentration *versus* A_{550}) the NO levels (micro molar level) in the samples were estimated.

2.1.7. RNA extraction and cDNA synthesis

Total RNA was isolated from all the PBMCs collected during the recovery time using Cold trizol (Sigma-Aldrich, USA) method, following manufacturer recommendations. The samples were treated with DNase (RNase-free DNase Set, Qiagen) to remove contaminating genomic DNA. The extracted RNA was evaluated spectrophotometrically. The integrity of the RNA was checked by visualization of 18 s and 28 s ribosomal bands on an agarose gel.

RNA was reverse transcribed using ProtoScript first strand cDNA synthesis kit (New England Biolabs, Beverly, MA, USA) as per the manufacturer's recommendations using random primers. The cDNA products were stored at –20 °C. An equal concentration

Table 1
Primers used for the present study.

Gene	Primer sequence	Size (bp)
HSP70	F: AACATGAAGAGCGCCGTGGAGG R: GTTACACACCTGCTCCAGCTCC	171
GAPDH	F: TGCCATCACAGCCACACAGAAG R: ACTTTCCCCACAGCCTTAGCAG	123