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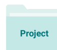
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ORIGINAL ARTICLE

Comparative studies on temperature threshold for heat shock protein 70 induction in young and adult Murrah buffaloes

N. Haque[1], A. Ludri[1], S. A. Hossain[2] and M. Ashutosh[1]

1 Dairy Cattle Physiology Division, National Dairy Research Institute, Karnal, Haryana, India, and
2 Dairy Cattle Nutrition Division, National Dairy Research Institute, Karnal, Haryana, India

Keywords
heat shock protein 70, physiological parameters, heat stress, young and adult Murrah buffalo

Correspondence
N. Haque, Dairy Cattle Physiology Division, National Dairy Research Institute, Karnal, Haryana 132001, India. Tel: +91-7404129829; E-mail: haquenilufar@gmail.com

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Summary

To know the temperature threshold for heat shock protein 70 (HSP70) induction in lymphocytes and to assess physiological changes, if any, in relation to HSP70 induction in young and adult Murrah buffaloes, this study was divided into two parts: I. *In vivo* study: where assay of HSP70 was performed in blood samples collected from acutely exposed young and adult Murrah buffaloes (*n* = 6) inside a climatic chamber at 40, 42 and 45 °C for 4 h and thermoneutral temperature (22 °C). Physiological parameters *viz.*, rectal temperature, respiratory rate, pulse rate and skin temperature of different body parts were monitored to assess magnitude of stress in the animals owing to thermal exposure II. For *in vitro* study, equal numbers of lymphocyte cells were separated from blood collected from young and adult buffaloes and were subjected to four temperature treatments (38, 40, 42 and 45 °C) for 4 h. A significant increase ($p < 0.05$) in all the physiological parameters in both young and adult buffaloes was observed after exposure to 40, 42 and 45 °C for 4 h as compared to 38 °C. The average plasma HSP70 concentrations (ng/ml) were significantly higher ($p < 0.05$) at 40, 42 and 45 °C as compared to 38 °C in both young and adult and were higher in young than adult buffaloes at 38 and 45 °C. Heat shock protein 70 level in lymphocyte lysate showed highest concentration after 3-h exposure to all temperatures (40, 42 and 45 °C) in both young and adult buffaloes. The intensity of changes of all physiological parameters was more in young animals than in the adults indicating the greater susceptibility of younger animals to heat stress and was found to be changed at around 40 °C when animals were exposed to different temperatures, indicating the possibility that HSP70 production may be initiated at this temperature which is 2 or 3 °C higher than core body temperature.

Introduction

India has approximately 97 million buffaloes, which represents 56.5% of the world buffalo population. India is the first country in the world for number of buffaloes and milk production (approximately 55 million tons) and possesses the best milch breeds. Among these, Indian Murrah is the most important and well-known buffalo breed in the world. Like all other mammals, buffaloes are homeotherms *i.e.* they maintain a constant body temperature by constantly regulating peripheral and internal body temperature with the assistance of cutaneous sensors and internal temperature sensors (located in the hypothalamus) along with integration of the endocrine system. Tissue, cellular metabolism and the underlying

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biochemical reactions that sustain life and productive functions need body temperature to be maintained within very narrow limits. An increase in body temperature of around 1.0 °C may result in detectable, deleterious effects on metabolism, tissue integrity and a significant depression in production (McDowell et al., 1976; Shebaita and El-Banna, 1982).

Exposure to high ambient temperature is the major constraint on buffalo productivity in hot climatic areas. The reduction in productivity with devastating economic consequences to the global dairy industry owing to warm environment has been documented (Bernabucci et al., 2010). In stressful conditions, a number of physiological and behavioural responses vary in intensity and duration in relation to the animal genetic make-up and environmental factors. Increasing air temperature above critical threshold is related to reduced feed intake, decreased activity and milk yield (Umphrey et al., 2001) and a deleterious effect on the physiologic status of farm animals (West, 2003). Such changes result in impairment of productive and reproductive performance of animals and buffaloes in particular (Habeeb et al., 2007).

The ability to adapt to physiological stress is of great importance to all living organisms, including domestic livestock. At the cellular level, cells respond to stresses by the production of a specific set of proteins called heat shock or stress proteins (Craig, 1985; Lindquist, 1986). Heat shock proteins (HSPs) are present in both prokaryotic and eukaryotic cells. Heat shock proteins represent between 2% and 15% of total cellular protein (Morimoto et al., 1994). These proteins allow cells to adapt to gradual changes in their environment. Heat shock proteins have been observed in every cell type and tissue, under both unstressed and stressed conditions. Heat shock proteins are present in the cytosol, mitochondria, endoplasmic reticulum and nucleus. They typically have a relatively long half-life. The degree of induction depends on the level and duration of exposure to stress (Kiang and Tsokos, 1998).

HSPs are induced in cells exposed to sublethal heat shock. Thermal stress-induced HSPs in cells are often used as markers of stress and adaptation in a variety of physiological systems. The stress proteins belong to a multigene family and range in size from 8 to 150 kDa. Heat shock protein 70 is the most highly inducible HSP after thermal stress (Beckham et al., 2004) and the most widely studied of all the HSPs, reported to have a number of important chaperoning functions, including aiding in the folding of new proteins, refolding of incorrectly folded proteins, reducing protein aggregates, presenting proteins in a conformation suitable for degradation by the proteasome and presenting steroid receptors in a ligand-binding conformation (Fink, 1999; Collier et al., 2008). Heat shock protein 70 is expressed in a differential manner; both constitutively and following heat shock, by ovine and bovine leucocyte subpopulations (Agnew and Colditz, 2008). Heat shock protein 70 are highly conserved and demonstrate a 60–78% base identity among eukaryotic cells (Caplan et al., 1993).

HSP70 could serve as a prognostic marker in heatstroke (Dehbi et al., 2010). In addition to this function, HSPs exert an immune-stimulatory effect, activate the host inflammatory response (Johnson and Fleshner, 2006), increase in the phagocytosis and maturation of phagosomes (Anand et al., 2010), in the modulation of the autoimmunity and tumour immunity (Alam et al., 2009). Not only that, HSP expression has also been detected in gametes and early-stage embryos (Wilkerson and Sarge, 2009). Those reports suggest that HSP gene expression is related to embryonic survival and overall pregnancy success.

This study helps us in understanding the role of HSP70 in young and adult buffaloes. Keeping the above points in view, the present research work was conducted with the objectives: (i) To know the temperature threshold for HSP70 induction in lymphocytes of young and adult Murrah buffaloes. (ii) To assess physiological changes, if any, in relation to HSP70 induction in buffaloes.

Materials and methods

Selection, feeding and maintenance of animals

Two groups of healthy Murrah buffaloes, young (1–2 years) and adult (3–4 years) were selected for the experiment. Each group contains six animals (total number of animals used = 12). The experimental animals were maintained as per standard practices followed at the institute of National Dairy Research Institute, Karnal, India, for young and adult buffaloes. This consists of feeding *ad lib* roughages and water as per Kearl (1982) feeding standard. Concentrate mixture was fed at 1 kg/animal. Concentrate mixture consisted of mustard cake, maize, wheat bran, rice bran, mineral mixture and salt. The CP% of diet was 12%, and TDN was 60%.

Exposure to climatic chamber

A climatic chamber (22′26′′ × 10′10′′ × 8′) insulated thermostatically fitted with heat convector was used

for exposing animals. The temperature of chamber was maintained at 40.0 ± 1.0 °C, 42.0 ± 1.0 °C and 45.0 ± 1.0 °C prior to experiment. The average relative humidity was 50% (average T_{db} = 42 °C and T_{wb} = 36 °C) inside the climatic chamber. All the animals (three at a time) were exposed to 4 h at above-mentioned different temperatures inside the chamber. Three-day interval was maintained for the exposed animals in between two temperature treatments to nullify the effect of earlier acute heat stress. Animals used in each group of temperature treatment are six, and the total number of animals used for this experiment is 12. After 4-h exposure, various physiological reactions were measured and blood samples were collected for laboratory study.

Experiment-I (*in vivo*)

Physiological parameters

Different physiological parameters *viz.* respiration rate, pulse rate, rectal temperature, skin temperature of different body parts *i.e.* head, hump, mid-dorsal, left thigh and right thigh of animals were recorded after 4-h exposure at all the above-mentioned temperatures inside the climatic chamber. Physiological parameters were also measured from the same animals in the month of March when the average environmental temperature was approximately 22 °C, which is considered to be the temperature of thermoneutral environment for tropical animals and used as control. Skin temperature was recorded using a non-contact temperature measurement instrument (RaytekR, model Raynger ST 2L, Santa Cruz, California, CA, USA).

Collection of blood sample

Blood samples were collected for laboratory study after exposing the animals at above-mentioned temperatures for 4 h inside the climatic chamber and were also collected in March when environmental temperature was 22 °C (thermoneutral environment) and used as control. Approximately 10 ml blood was drawn in sterile vacutainer tubes containing EDTA (1 mg/ml) anticoagulant from jugular vein puncture posing minimum disturbances. Immediately, the tubes were brought to the laboratory for plasma separation, which was performed by centrifugation of the blood sample at 210 ***g*** for 30 min. Separated plasma was stored at −20 °C till estimation of HSP70.

Estimation of plasma HSP70

Estimation of plasma HSP70 was performed by modified method of Gutierrez and Guerriero (1991).

Assay protocol

The assay polystyrene plates were prepared by adding 20 ng/well of purified HSP70 (obtained from bovine brain procured from Sigma-Aldrich, St. Louis, MO, USA) in 100 μl carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$ and 30 mM NaN_3, pH 9.6) to 96-well microtiter polystyrene plate (Nunc Poly-Sorp, Thermo Scientific, Rochester, NY, USA) and allowed to incubate overnight at 4 °C. For the blank wells, coating was performed with carbonate buffer alone. Heat shock protein 70 standards were prepared by diluting it to a final stock concentration of 800 ng/100 μl of antibody buffer (10 mM Tris (pH 7.4), 0.15 M NaCl, 30 mM NaN_3 and 1% Triton X-100] and were serially diluted to a lowest concentration of 3.125 ng/100 μl. On the same day, 100 μl of sample and 100 μl of diluted (1:2000) monoclonal HSP70 antibody [produced in mouse (clone BRM-22, ascitis fluid; Sigma-Aldrich)] were added in 1.5-ml microcentrifuge tubes and allowed to incubate overnight at 4 °C. Similarly, 100 μl of each different standard was mixed with 100 μl of diluted monoclonal HSP70 antibody and allowed to incubate overnight at 4 °C. On the following day, the contents of the plates were thrown away and washed five times with washing buffer or Tris-buffered saline (TBS). Three hundred microlitres of blocking buffer [To 500 ml of Tween 20 Tris-buffered saline (0.605 g of 10 mM Tris (pH 7.4); 4.38 g of 0.15 M NaCl; and 250 μl of 0.05% Tween 20), 1.25 g of 0.25% porcine skin gelatin was added and was slightly warmed to hasten dissolution of gelatin] was added to each well and kept for 25 min at room temperature. Again, washing was performed five times with 300 μl of washing buffer each time. One hundred microlitres of the mixture containing antibody and sample and antibody and standard was added to each well in duplicates. In maximum binding wells, 100 μl of antibody without any standard and sample was added. In non-specific binding wells (NSB), 100 μl of antibody buffer alone was added. The plates were incubated overnight at 4 °C. Next day, the contents were decanted and washed five times with 300 μl of washing buffer. The plates were then incubated with 100 μl of diluted secondary antibody (goat anti-mouse IgG conjugated with alkaline phosphatase diluted with blocking buffer at 1:2000 *i.e.* 25 μl in 50 ml) for 2 h at room temperature. The contents were then decanted after 2 h and then washed five times with 300 μl of washing buffer. One hundred μl of freshly made *p*-nitrophenyl phosphate (PNPP, 1 mg/ml) in substrate buffer (100 mM triethanolamine, 1 mM $MgCl_2$ and 30 mM NaN_3, pH 9.8) was then added to each well and

incubated at 37 °C for 4 h (time required by the maximum binding to develop the colour with OD −0.9) or until absorbance. The reaction was then stopped with 2 M NaOH. The plates were then read at 405 nm in an ELISA plate reader. The standard curve was prepared in spreadsheet using MS-EXCEL. The concentration of HSP70 of each sample in each plate was calculated from respective standard curve.

Experiment-II (*in vitro*)

Blood sample was collected from unexposed buffaloes in both age groups (young and adult, $n = 6$), and the lymphocyte cells were separated through density gradient centrifugation method.

Lymphocyte cell separation through gradient centrifugation method

Fifty millilitre blood was collected in heparinized tubes from any of the one unexposed animal and diluted blood in the ratio of 1:2 in serum-free media. Whole blood was layered carefully onto the histopaque (1.007 density) using a pasteur pipette to produce a clean interface between the two layers. To obtain the maximum yield, the proportion of blood to histopaque used was in a ratio of 1:3 *i.e.* 3 ml of blood mixed media layered onto 7 ml histopaque. Further, it was centrifuged at 30 300 ***g*** for 30 min at room temperature. The white opaque mononuclear fraction from the interface was collected between the diluents and the histopaque, added at least five volume of serum-free medium and further centrifuged at 210 ***g*** for 10 min for washing. Following was repeated for two more times to remove histopaque. Finally, the pellet obtained was diluted with serum-free RPMI-1640 media, and the cell number was counted in a hemocytometer using trypan blue (0.4%) to know the number of cells.

The lymphocytes obtained as above were further divided into thirteen equal parts in flasks after counting. The lymphocyte cells were incubated at 38, 40, 42 and 45 °C (four flasks for each temperature) for 4 h. The lymphocytes were taken out from the incubator at every 1-h interval. Further, the lysate was prepared from the pellet using hypotonic buffer.

Preparation of cell lysate

Hypotonic buffer (10 mM $NaHCO_3$, 0.5 mM PMSF) was prepared by dissolving 0.86 g of $NaHCO_3$ and 0.08 g of PMSF in autoclaved MilliQ water (Milli Q Biocel, Millipore SAS, Molsheim, France). The pH was adjusted to 7.1. Hypotonic buffer was added to cell pellets for sonication approximately at 25×10^6 cells/ml. The tubes were then kept on ice for at least half an hour before sonication. Sonication was carried out at 20 Branson power output for 3 min at break of 1 min for every 1 min. Cell lysates were centrifuged at 21 040 ***g*** for 30 min. The supernatants were collected and preserved at −20 °C until ELISA was carried out. Heat shock protein 70 was estimated from this lysate by modified method of Gutierrez and Guerriero (1991) as described earlier.

Statistical analysis

All the data generated were statistically analysed by two-way analysis of variance (ANOVA) using SigmaStat (11.0 software package) Statistical Analysis System (Jandel Scientifiic, San Rafael, CA, USA), according to Snedecor and Cochran (1994). Data from different experiments are presented graphically as mean ± SE. Differences were considered to be significant at $p \leq 0.05$.

Results

In vivo Study

Effect of thermoneutral and different controlled climatic temperatures on different physiological parameters in young and adult Murrah buffaloes

All physiological parameters of experimental animals are presented in Table 1. There was significant increase ($p < 0.05$) in rectal temperature at 40, 42 and 45 °C groups as compared to 22 °C in both young and adult buffaloes. The temperature interaction between 40 and 45 °C; 42 and 45 °C showed a significant effect ($p < 0.05$) on rectal temperature in both age groups, but the rectal temperature at 40 and 42 °C was statistically similar. On comparison of two age groups, it was found that only at 22 °C, rectal temperature was significantly higher ($p < 0.05$) in young buffaloes whereas at other temperatures, it was statistically similar in both groups.

In case of respiration rate in young animals, the rate was statistically different ($p < 0.05$) with each other at the above temperatures in both young and adult. But in adult animals, at 42 and 45 °C, the respiration rate was statistically similar. In different age groups, it was found that there was no significant difference ($p < 0.05$) in respiration rate at the above temperatures except in case of 45 °C, and significant higher ($p < 0.05$) respiration rate was observed in young animals than in adults.

In both young and adult animals, the increasing temperature had significant effect ($p < 0.05$) on

Table 1 Comparisons of different physiological parameters in young and adult Murrah buffaloes exposed to different temperatures

Parameters	Young				Adult				Significance of effects (p)			
	Temperature (°C)											
	22	40	42	45	22	40	42	45	Age	Temp.	Age × temp.	SEM
Rectal temperature (°C)	38.48^{ay}	39.15^{bx}	39.22^{bx}	39.74^{cx}	37.20^{ax}	38.91^{bx}	38.97^{bx}	39.53^{cx}	<0.001	<0.001	<0.001	0.102
Respiration rate (counts/min)	13.67^{ax}	61.67^{bx}	69.67^{cx}	86.00^{dy}	13.33^{ax}	57.00^{bx}	68.67^{cx}	69.17^{cx}	0.042	<0.001	<0.001	1.823
Pulse rate (counts/min)	42.67^{ax}	62.33^{by}	75.33^{cy}	76.00^{cx}	44.33^{ax}	58.67^{bx}	70.00^{cx}	75.33^{dx}	0.015	<0.001	0.015	1.114
Skin temp. (°C)												
Head	32.57^{ax}	37.25^{bx}	38.25^{cx}	38.40^{cx}	32.78^{ax}	37.33^{bx}	38.73^{cx}	38.82^{cx}	0.121	<0.001	0.872	0.268
Hump	34.13^{ax}	39.28^{by}	39.30^{by}	39.60^{bx}	35.43^{ay}	38.30^{bx}	38.32^{bx}	39.70^{cx}	0.503	<0.001	<0.001	0.296
Middorsal	33.12^{ax}	38.13^{bx}	38.80^{bcx}	39.20^{cx}	34.68^{ay}	37.93^{bx}	38.52^{bcx}	38.93^{cx}	0.230	<0.001	<0.001	0.237
Left thigh	34.68^{ax}	37.90^{bx}	38.10^{bx}	38.32^{bx}	35.02^{ax}	38.20^{bx}	38.17^{bx}	38.72^{bx}	0.114	<0.001	0.908	0.240
Right thigh	34.62^{ax}	38.10^{bx}	38.30^{bx}	38.37^{bx}	34.73^{ax}	38.32^{bx}	38.80^{bx}	38.92^{bx}	0.041	<0.001	0.742	0.232

Means with different superscripts in rows for a parameter differ significantly (p < 0.05).
^{a,b,c,d}Significant difference between temperature; ^{x,y}significant difference between age group.

pulse rate. The different temperatures showed a significant statistical difference ($p < 0.05$) in pulse rate with each other except between 42 and 45 °C in young animals. On comparison of effect of age on pulse rate, it was found that there was significantly higher pulse rate ($p < 0.05$) at 40 and 42 °C in young animals as compared to the adults where 22 and 45 °C showed no significant difference ($p < 0.05$) in between these two age groups.

In all regions, the skin temperatures showed an increasing trend with increasing temperatures although in some of the cases they were not statistically significant ($p < 0.05$).

Effect of thermoneutral and different controlled climatic temperatures on plasma HSP70 concentration (ng/ml) in young and adult Murrah buffaloes

The average plasma HSP70 concentrations (ng/ml) were 35.09 ± 1.49, 49.46 ± 4.46, 49.70 ± 2.13 and 63.43 ± 4.27 in young animals and 26.20 ± 1.34, 45.04 ± 2.82, 49.90 ± 3.31 and 54.07 ± 3.35 in adult animals at 22, 40, 42 and 45 °C temperatures, respectively (Fig. 1). The results showed that the plasma HSP70 concentrations were statistically higher ($p < 0.05$) in 40, 42 and 45 °C as compared to 22 °C in both the groups. The plasma HSP70 concentration was also statistically higher ($p < 0.05$) at 45 °C as compared to 40 and 42 °C in young buffaloes. In adult buffaloes, the plasma HSP70 concentrations were statistically similar ($p < 0.05$) at 40, 42 and 45 °C. Among age groups, it was found that the plasma HSP70 concentrations were statistically higher ($p < 0.05$) in young animals as compared to adults in 22 and 45 °C temperature groups. Plasma HSP70 concentrations were statistically similar in 40 and 42 °C

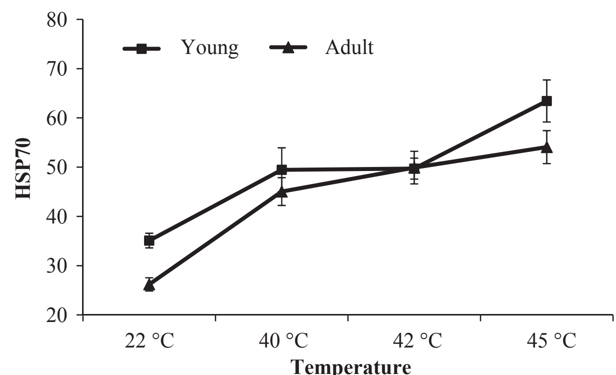

Fig. 1 Plasma heat shock protein 70 (HSP70) concentration (ng/ml) in young and adult Murrah buffaloes exposed to different temperatures. Bars indicate SE. Significance of effects for age, p = 0.013; temp, p < 0.001; age × temp. interaction p = 0.375.

in young as well as in adult animals. There was a significant effect of age group and temperature to which the animals were exposed on the level of plasma HSP70. The effect of different temperatures on plasma HSP70 does not depend on the age group of animal. There is not a statistically significant interaction between temperature and age group.

Interrelationship of different physiological reactions with HSP70

It was observed that coefficient of determination (R^2) of rectal temperature and HSP70 was 0.9932 in young buffaloes (Fig. 2) and 0.9807 in adult buffaloes (Fig. 3). The R^2 between respiration rate and HSP70 were 0.9129 and 0.9796 and in case of pulse rate and HSP70 were 0.7754 and 0.9459 in young and adult animals, respectively. All the R^2 values

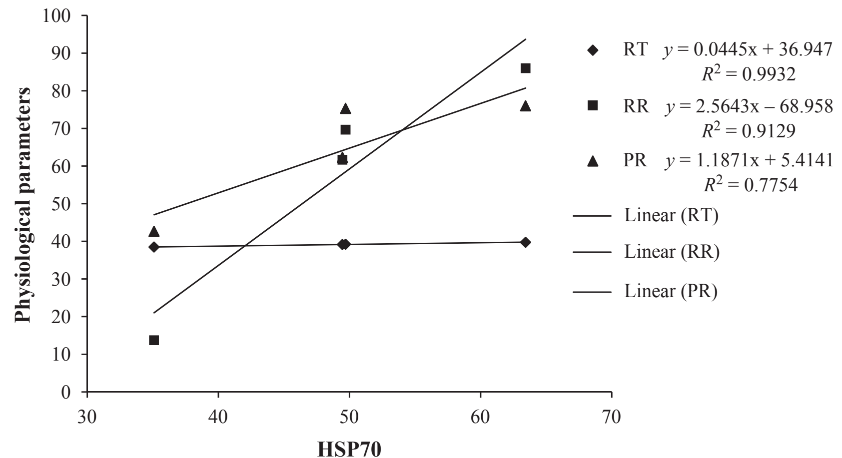

Fig. 2 Linear regression graph between heat shock protein 70 (HSP70) concentration and rectal temperature (RT), respiration rate (RR), pulse rate (PR) in plasma in young Murrah buffaloes.

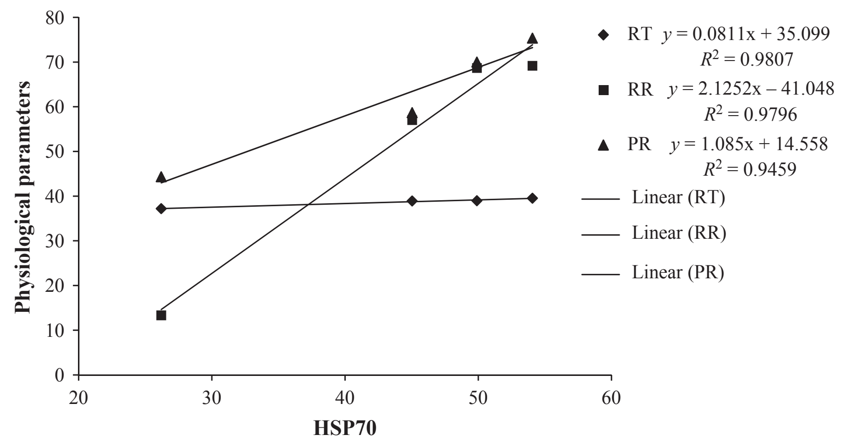

Fig. 3 Linear regression graph between heat shock protein 70 (HSP70) concentration and rectal temperature (RT), respiration rate (RR), pulse rate (PR) in plasma in adult Murrah buffaloes.

showed a high positive interrelationship between the physiological parameters with HSP70. Thus, the physiological reactions and HSP70 can be considered as classical indicator of stress.

In vitro study

Effect of different temperatures on HSP70 level (ng/million cells) of lymphocyte lysate in young and adult Murrah buffaloes

The average levels of HSP70 in lymphocyte lysate exposure to different temperatures in young and adult Murrah buffaloes at different intervals are summarized in Table 2. The average levels of HSP70 (ng/million cells) in lymphocyte lysate were significantly higher in young Murrah (28.65, 41.21, 55.31 and 38.48) as compared to adult animals (24.03, 35.02, 50.07 and 35.53) when lymphocyte pellets were exposed to 38, 40, 42 and 45 °C for 4 h, respectively. In young and adult buffaloes, the highest level of HSP70 was found to be at 42 °C. The levels of HSP70 were also significantly higher at 40 and 45 °C compared to 38 °C in both young and adult, but the intensity was not so high as in 42 °C. After 42 °C, there was a decline at 45 °C. The different level of temperature and the duration of time at which the lymphocyte lysates were exposed have significant effect on the HSP70 concentration in lymphocyte lysate in both young and adult animals. The effect of different levels of temperature on HSP70 concentration in lymphocyte lysate depends on how much time the lymphocytes were exposed. There is a statistically significant interaction between temperature and hour.

In young and adult buffaloes, there was an increasing trend in level of HSP70 with the advancement of hour and highest levels were found after 3-h exposure in both groups. After 3 h, there was a decline in the HSP70 levels. The effect of different temperatures on plasma HSP70 does not depend on

Table 2 Average level of heat shock protein 70 (ng/million cells) in lymphocyte lysate in young and adult Murrah buffaloes exposed to different temperatures for different hours

Hours	Young				Adult				Significance of effects (p)							
	Temperature (°C)															
	38	40	42	45	38	40	42	45	Age	Temp.	h	Age × temp.	Age × h	Temp. × h	Age × temp. × h	SEM
1h	28.81^{ax}	41.13^{cx}	49.70^{dx}	33.82^{bx}	24.07^{ax}	33.64^{bx}	46.04^{cx}	33.40^{bx}								
2h	27.59^{ax}	40.11^{bx}	57.64^{yzc}	39.26^{bxy}	23.84^{ax}	36.01^{bx}	51.84^{cxy}	37.18^{bxy}								
3h	28.99^{ax}	45.11^{bx}	61.88^{cz}	43.32^{by}	24.01^{ax}	37.68^{bx}	55.21^{cy}	40.68^{by}								
4h	29.20^{ax}	38.51^{bx}	52.01^{cxy}	37.53^{bxy}	24.21^{ax}	32.74^{bx}	47.20^{cx}	30.87^{bx}								
Overall Mean	28.65^{xn}	41.21^{yn}	55.31^{zn}	38.48^{ym}	24.03^{xm}	35.02^{ym}	50.07^{zm}	35.53^{ym}	<0.001	<0.001	<0.001	0.289	0.68	<0.001	0.880	0.304

Means with different superscripts in rows for a parameter differ significantly ($p < 0.05$).
^{a,b,c,d}Significant difference between temperature; ^{x,y,z}significant difference between hour; age group; ^{m,n}significant difference between age group.

the age group of animal. There is not a statistically significant interaction between temperature and age group.

Discussions

The results of physiological parameters are in agreement with the observations of Hales (1973), Tinh et al. (1996), Das et al. (1999), Koga et al. (2004), Sevegnani et al. (2007), Thanh and Chang (2007), Gudev et al. (2007), Patir and Upadhyay (2007), Mayengbam (2008) and Lallawmkimi (2009). However, in case of pulse rate, Kibler and Brody (1951) and Singh and Newton (1978) reported that with increase in temperature there was a decline in pulse rate. But, Huhnke and Monty (1976) did not find any significant variation in the pulse rate of pre- and post-parturient Holstein–Friesian cows during the cool and hot weather seasons.

The great changes in RT, RR and PR are induced in buffaloes by a marked increase in blood flow from the body core to the surface, which accelerates dissipation of heat from the skin surface. The increase in respiration rate with the increasing temperature may be due to the more demand of oxygen by the tissues in stressful condition. The redistribution of cardiac output generally arises from the necessity to dissipate heat under hot conditions, which is facilitated by sending more blood to the surface of the body. The excessive heat is left in the subcutaneous tissues, and skin temperature increases. Consequently, the internal heat gradient between the rectal and skin temperature is narrowed, and the external heat gradient between the skin and the surrounding air is widened, thus promoting body heat dissipation from the skin surface. Heat stress causes cutaneous vasodilatation which causes a rise in skin temperature, which ultimately steepens the thermal exchange gradient for environmental temperatures below skin temperature.

The observations of the present *in vivo* study for HSP70 are in agreement with those of Kristensen et al. (2004) who reported that the HSP70 concentration was the highest in old heifers and lowest in cows early in lactation and cows late in lactation and effect of age was highly significant. These findings support our observations as the HSP70 concentration was statistically higher ($p < 0.05$) in young animals as compared to adults at 22 °C, which is the thermoneutral zone. An increase in HSP70 indicates a lower tolerance of extreme heat exposure and is associated with higher expression of HSP70. In our study, higher level of HSP70 in young animals indicates that these

animals are more susceptible to heat stress as compared to their adult counterpart.

The findings of increasing concentration of HSP70 with increasing temperature are in collaboration with the findings of Javid et al. (2007), Wang et al. (2008), Cao et al. (2009), Patir and Upadhyay (2010), Mishra et al. (2011). The decline in HSP70 concentration of lymphocytes at 45 °C has been observed in both young and adult buffaloes that may be attributed to decrease in viability of lymphocytes and as a consequence of HSP70 induction. Wang et al. (2001) found no detectable HSP70 expression at low temperature than 41 °C *i.e.* at 37 °C. But the heating of cells at 44 °C for 2 h caused a decline in HSP70 content because of lethal thermal effect on cells. Similar results have also been reported by Valenzuela (2000) on lymphocytes of five different cattle breeds at 40, 42 and 44 °C for 4 h. A decrease in HSP70 expression at 44 °C was observed. The changes observed *in vivo* study with respect to physiological functions and HSP70 were most probably associated with the level of stress experienced earlier or acute stress to acquired thermotolerance. The greater the initial heat exposure and the magnitude, higher will be the thermotolerance. Another possible explanation for thermal changes is that a destabilizing protein exists in heat shocked non-thermotolerant cells that interacts with HSP70 mRNA and declines its induction, while the interaction is protected in thermotolerant cells (Nicholas et al., 1999). Currie and White (1983) have also indicated that a core temperature of 42 °C for 20–60 min stimulated the production of HSPs. But if the core temperature is raised to 40 °C or higher and if leucocytes are exposed to 41 °C, it reduces the subsequent secretion of HSP70.

Presence of increased level of HSP70 during thermal stress indicates that during thermal stress certain non-native proteins are produced in the cells that are toxic to the cells. To combat this stressful situation, there is induction of more HSP70 which binds the non-native protein that are processed by macrophages as foreign antigens for presentation to lymphocytes.

Prior to sudden exposure of cells to high temperature, the lymphocyte may be at some degree of stress and HSP70 might have started expressing in those cells, a sudden exposure of cells to 40, 42 and 45 °C for 4 h potentiated the synthesis of HSP70 in lymphocytes of buffaloes. Wang et al. (2003) also reported that HSP70 expression kinetics is a couple function of heating temperature, time and post-heating duration. Maximum HSP70 expression of approximately 10 times the basal level has been observed by Wang et al. (2003) at approximately 1.6 h of effective heating at 42 °C in bovine aortic endothelial cells. The heated bovine aortic endothelial cells at different temperatures and durations in culture dish indicated that at 42 °C, the HSP70 expression is more than 8 times larger than control and 10 times at 43 °C, but, at 44 °C, cells become rounded and detached from culture dish, indicating cell necrosis. Patir and Upadhyay (2007) found in their study that HSP70 levels were significantly higher in cells primed before exposure that indicated a threshold of thermal dose for maximum HSP70 expression in buffaloes. It has therefore evidenced that buffaloes that have evolved in hot climates might have acquired genes that protect cells from the deleterious actions of elevated temperature.

Conclusions

Heat stress in farm animals, such as cattle and buffalo during summer and post-summer seasons, is a problem for livestock producers. The effect of heat stress becomes pronounced when heat stress is accompanied with ambient humidity impairing the immune status, growth, production and reproductive performance of animals. Lymphocytes isolated from buffaloes were heat stressed *in vitro* and responded by synthesizing thought to help the cell survive this stress. This cellular response may be an important mechanism by which these animals respond to heat stress. Heat shock protein 70 levels may be used as an important and effective biomarker for heat stress management in livestock animals.

In conclusion, we are currently pursuing the hypothesis that the intensity of changes of all the physiological parameters was more in young animals than the adults indicating the greater susceptibility of younger animals to heat stress. Finally, all the physiological parameters were found to be changed at around 40 °C when animals were exposed to different temperatures indicating the possibility that the threshold temperature for HSP70 production may be 40 °C which is 2 or 3 °C higher than core body temperature. Further research is needed, to determine the exact threshold temperature in which HSP70 induction will be occurred in buffaloes using narrow temperature range below 40 °C.

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