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HSP 72 expression and antioxidant enzymes in Murrah buffaloes during heat exposure in climatic chamber

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ABSTRACT

Present investigation was undertaken to monitor the level of HSP-72 mRNA expression in lymphocyte and antioxidant enzymes in RBC lysate during exposure in climatic chamber. Six each of growing (6–12 months), heifer (2–2.5 years) and lactating (2–3parity) Murrah buffaloes were selected from the NDRI herd and exposed at 2 different conditions, viz. exposure I (38±1°C with 50±2%RH) and exposure II (42±1°C with 40±2% RH) in climatic chamber for 3 h continuously. Blood samples were collected prior to exposure, at hourly interval during exposure and after end of 3 h of exposure. The levels of erythrocyte SOD and catalase increased significantly in all experimental groups after 3 h of exposures. The magnitude of increase in SOD and catalase was significantly higher at exposure II compared to exposure I in all groups of animals. The HSP72 mRNA expressions increased significantly after end of 3 h of exposures in growing and heifers of Murrah buffaloes during both exposure I and II whereas in lactating buffaloes the HSP72 mRNA expressions increased during first 2 h of exposures and later on a declining trend was observed at third hour. The magnitude of increase in HSP 72 mRNA expression was significantly higher during exposure II compared to exposure I in all groups of animals. The levels of HSP 72 mRNA expression and antioxidant enzymes increased with the increase in temperature indicating an oxidative stress. Therefore, the Murrah buffaloes need to be protected from extreme heat stress (42°C) to maintain homeostasis and oxidative status within the physiological limits to sustain the productivity.

Key words: Antioxidant enzymes, Climatic chamber, HSP 72 expression, Murrah buffalo

Temperature and humidity, the key environmental factors, affect organisms from the cellular to organism level through physiological and biochemical processes. Due to changing climatic conditions, a more lucid understanding of the mechanisms of thermal tolerance in livestock species has become imminent (Harley *et al.* 2006, Crozier *et al.* 2008. In the past years, attempts had been made to find out the levels of thermotolerence of cattle and buffaloes based on physiological and cardinal reactions. The levels of animal's heat tolerance can be assessed by a group of protein family known as Heat Shock Proteins (HSP). These proteins are highly conserved cellular stress proteins in organism from bacteria to man. Stressful conditions in animals elicit HSP synthesis especially the HSP 70. HSP 70 function as molecular chaperones in restoring cellular homeostasis and promoting cell survival (Horowitz 2001, Collier *et al.* 2008).Therefore intracellular levels of HSP were suggested as indicators of thermotolerence in livestock species. Under stressful conditions, free radicals are produced in the bodies which are neutralized by interacellular antioxidant enzymes produced in the different tissues of the body depending upon their metabolic activity. Free radicals are generated in the body during normal metabolic process and their concentration increased under stressful conditions. Oxidative stress is a pathological state that results from imbalance production of reactive oxygen species and antioxidant defense enzymes to convert ROS to less harmful molecules. Oxidative stress alters important physiological and metabolic functions of the body (Gitto *et al.* 2002, Grummer and Mashek 2004).

Buffaloes have poor heat tolerance and need protection against adverse climatic conditions. The mechanism of heat tolerance in buffaloes has been poorly understood by studying morphological, behavioral and physiological characteristics

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i.e. respiratory frequency, rectal temperature, sweating and panting abilities during acute and long-term heat exposures. Keeping the above points in view, the present study was undertaken to monitor the levels of antioxidant enzymes and heat shock protein 72 expression in lymphocytes during exposures in climatic chamber at 40 and 42°C.

MATERIALS AND METHODS

Six animal each of growing (6–12 months), heifer (2–2.5 years) and lactating (2–3parity) Murrah buffaloes were selected from NDRI, Karnal herd. These animals were exposed in a climatic chamber at 38±1 °C with 50±2% relative humidity (RH) and at 42±1°C with 40±2% RH for 3 h continuously. All these animals were fed ad libitum roughages (green and dry) as per the practices followed at farm (Kearl 1982) and water was available round the clock. The concentrate ration for maintenance and productive processes were provided to all the experimental animals. The concentrate mixture consists of mustered cake, maize, wheat bran, rice bran, mineral mixture and salt. Blood samples were collected prior to exposure, at hourly interval during exposure and after end of 3 h of exposure. Antioxidants enzymes, viz. superoxide dismutase (EC 1.15.1.1) and catalase (EC 1.11.1.6) were estimated in the RBC lysate as per the methods of Marklund and Marklund (1974) and Aebi (1984) respectively. The RBC lysate pellets were washed and centrifuged thrice with normal saline (0.9% NaCl) solution. Then distilled water was added to pellet slowly with constant stirring up to the marked level and the lysate was quickly stored at –20 °C till activities of enzymes (SOD and Catalase) were estimated.

Expression of HSP72 in peripheral blood leukocytes

Blood lymphocytes were separated by gradient centrifugation using Histopaque (specific gravity 1.077). Total RNA was extracted from lymphocyte using Tri reagent (Sigma) according to the manufactures instructions. The integrity of the RNA was checked by visualization of 18s and 28s ribosomal bands on an agarose gel (1.5%). Concentration of RNA was determined as displayed by NanoDrop in ng/îl while recording OD at 260nm and 280nm. From separated total RNA, cDNA was prepared by using Accuscript kit" (Stratagene) as per the manufactures instructions.

Primer was designed from *Bos taurus* heat shock 70 kDa protein 1A, mRNA sequence (NM_174550, HSPA1A, highly expressed HSP 70 member in blood leukocyte) as the buffalo sequences was not available. The obtained primers were used for amplification of cDNA from buffalo lymphocytes using Real-time PCR. The primer sequence for HSP72 was Forward-5'-GCTATCGGCATCGACCTG-3' and Reverse-5'-CTGGTTCTTGGCCGCATC-3'. The expected product size was 159bp and for the house keeping gene, GAPDH, Forward-5'-TCAAGAAGGTGGTGAAGCAG-3' Reverse-5'-CCCAGATCGAAGGTAGAAG-3' was used respectively and the expected product length was 121bp as shown in the Table 1.

Table 1. Primer sequences and amplicon size used for gene expression

Gene	Primer Sequence	Amplicon Size (bp)
HSP72	Forward- 5'-GCTATCGGCATCGACCTG-3'	
	Reverse- 5'-CTGGTTCTTGGCCGCATC-3'	159
GAPDH	Forward- 5'-TCAAGAAGGTGGTGAA GCAG-3'	
	Reverse- 5'-CCCAGATCGAAGGTAGAAG -3'	121

Statistical analysis: The data were analyzed by analysis of variance technique (Snedecor and Cochran 1968). Comparison between different treatments groups were carried by Duncan's multiple range test (DMRT) test. The difference at $P \leq 0.05$ was considered to be statistically significant. The following model was used for analysis:

$$Y_{ijkl} = \mu + T_i + S_j + D_k + e_{ijkl}$$

Where, $Y_{ijkl} = k^{th}$ observation of i^{th} temperature with j^{th} stage at k^{th} duration of exposures; μ, overall mean; T_i, effect of i^{th} temperature; S_j, effect of j^{th} stage; D_k, effect of k^{th} duration of exposure; and e_{ijkl}, random error.

RESULTS AND DISCUSSION

The level of antioxidant enzymes and HSP -72 mRNA expressions during different temperature exposures in different physiological stages of Murrah buffaloes have been presented in Table 2 and their statistical analysis in Table 3.

Antioxidant enzymes

Super oxide dismutase (EC 1.15.1.1): The SOD activity increased by 15.00, 7.11 and 21.10% over the initial values in growing, heifer and lactating buffaloes respectively after 3 h of exposure I. The respective increase at exposure II was significantly higher i.e. 33.75, 31.99 and 54.32% over the initial values in growing calves, heifer and lactating buffaloes respectively (Table 2). The level of erythrocytes SOD increase significantly ($P<0.05$) after three h of exposures in all the groups of the experimental animals during climatic chamber exposures and the magnitude of increase was found to be significantly ($P<0.01$) higher during exposure II compared to exposure I (Table 2). The level of SOD increased with the increase in the exposure h i.e., 1 to 3 h at both the exposures. Analysis of variance (ANOVA) of data for erythrocyte SOD activity revealed a significant ($P<0.05$) difference between stages, treatments, hours and the interaction between treatments×hours (Table 3).

The increase in erythrocytic SOD levels represent compensatory changes, the animal is undergoing in response to increased oxidative stress. The results of the present study are in general agreement with Kumar (2005) and Shibu

Table 2. Superoxide dismutase, catalase and HSP 72 mRNA levels at different physiological stages of Murrah Buffaloes subjected to heat stress in climatic chamber

Parameter	Exposure	Exposure period (hours)			
		Pre-exposure	1	2	3
		Growing calves			
SOD (U/min/g Hb)	38°C	2668.17 ± 38.89^{a}	$2731.51\pm53.02^{a}{}_{p}$	$2837.87\pm23.79^{b}{}_{p}$	$3070.38\pm33.13^{c}{}_{p}$
	42°C	2465.38 ± 2.99^{a}	$2837.92\pm48.91^{b}{}_{q}$	$3004.41\pm3.84^{c}{}_{q}$	$3298.39\pm3.63^{d}{}_{q}$
Catalase($\mu molH_2O_2$/min/mg Hb)	38°C	130.95 ± 1.63^{a}	$135.70\pm1.95^{b}{}_{p}$	$142.16\pm2.18^{c}{}_{p}$	$150.84\pm1.75^{d}{}_{p}$
	42°C	128.32 ± 0.36^{a}	$136.70\pm1.73^{b}{}_{p}$	$158.55\pm1.92^{c}{}_{q}$	$164.86\pm0.51^{d}{}_{q}$
HSP 72 mRNA	38°C	0.69 ± 0.02^{a}	$0.70\pm0.02^{ab}{}_{p}$	$0.74\pm0.04^{b}{}_{p}$	$0.72\pm0.02^{abc}{}_{p}$
	42°C	0.66 ± 0.06^{a}	$0.72\pm0.02^{b}{}_{p}$	$0.89\pm0.05^{c}{}_{q}$	$0.73\pm0.00^{bd}{}_{p}$
		Heifers			
SOD (U/min/g Hb)	38°C	2851.48 ± 33.96^{ab}	$2921.97\pm194.98^{ab}{}_{p}$	$2994.74\pm18.31^{bc}{}_{p}$	$3054.22\pm34.98^{d}{}_{p}$
	42°C	2605.54 ± 60.34^{a}	$2910.37\pm34.97^{b}{}_{p}$	$3045.63\pm39.17^{c}{}_{p}$	$3439.27\pm87.49^{d}{}_{q}$
Catalase ($\mu molH_2O_2$/min/mg Hb)	38°C	128.32 ± 0.37^{a}	$133.70\pm1.73^{bc}{}_{p}$	$135.55\pm1.92^{bc}{}_{p}$	$139.86\pm0.52^{cd}{}_{p}$
	42°C	125.88 ± 1.37^{a}	$139.05\pm0.67^{b}{}_{p}$	$148.19\pm0.49^{c}{}_{q}$	$155.99\pm1.55^{d}{}_{q}$
HSP 72 mRNA	38°C	0.54 ± 0.01^{a}	$0.68\pm0.01^{b}{}_{p}$	$0.72\pm0.06^{b}{}_{p}$	$0.72\pm0.02^{b}{}_{p}$
	42°C	0.52 ± 0.01^{a}	$0.75\pm0.04^{b}{}_{q}$	$0.86\pm0.06^{c}{}_{q}$	$0.74\pm0.02^{bd}{}_{p}$
		Lactating Buffaloes			
SOD (U/min/g Hb)	38°C	2641.05 ± 83.77^{a}	$2837.92\pm48.91^{b}{}_{p}$	$3004.41\pm53.84^{c}{}_{p}$	$3198.39\pm53.63^{d}{}_{p}$
	42°C	2598.56 ± 13.03^{a}	$2895.28\pm22.14^{b}{}_{q}$	$3833.78\pm16.09^{c}{}_{q}$	$4010.09\pm14.09^{d}{}_{q}$
Catalase ($\mu molH_2O_2$/min/mg Hb)	38°C	134.56 ± 1.95^{a}	$146.43\pm1.61^{b}{}_{p}$	$152.45\pm2.01^{c}{}_{p}$	$168.69\pm1.22^{d}{}_{p}$
	42°C	130.84 ± 1.32^{a}	$151.13\pm2.91^{b}{}_{q}$	$158.12\pm2.51^{c}{}_{q}$	$174.26\pm2.52^{d}{}_{q}$
HSP 72	38°C	0.62 ± 0.01^{a}	$0.73\pm0.05^{b}{}_{p}$	$0.72\pm0.01^{b}{}_{p}$	$0.63\pm0.01^{ac}{}_{p}$
	42°C	0.59 ± 0.09^{a}	$0.76\pm0.04^{b}{}_{q}$	$0.74\pm0.08^{c}{}_{p}$	$0.65\pm0.01^{d}{}_{p}$

The superscripts a,b,c,d differ significantly (P<0.05) in a rows and subscripts p,q differ significantly (P<0.05) in a column for each parameter.

Table 3**.** Analysis of variance for SOD and Catalase during different heat exposures in Murrah buffaloes

Source of variation	df	Mean sum of squares		
		SOD	Catalase	HSP72 mRNA
Stage	2	172,209.394**	197.767**	9.6341
Treatment	1	218,162.948**	2,140.142**	320.531**
Hours	3	2,233,802.164**	2,722.416**	294.524**
Stage×treatment	2	17,936.286	0.043	2.486
Stage×hours	6	33,479.104	15.966	3.433
Treatment×hours	3	442,690.935**	857.717**	129.989**
Stage×treatment×hours	6	58,431.411	22.532	1.693
Error	120	28,596.870	22.655	4.975

(2007) who reported a significant (P<0.05) increase in erythrocytic SOD activity in Murrah buffaloes, Karan Fries and Tharparkar cattle exposed to natural conditions. Jose *et al.* (2000) and Oztasan *et al.* (2004) also reported a significant rise in erythrocyte SOD activity in rats after stressful exercise. Ozturk and Gumuslu (2004) reported an increase in the levels of SOD in rats which were exposed to 40°C. In contrast to present study Shanmugham (2003) did not find any significant difference in the erythrocyte SOD activity in goats during warm (<29°C) and hot (>30°C) season. The probable reason may be that the goats are more adapted to high ambient temperature compared to buffaloes and exposure temperature was at lower magnitude. In present study, the increase in the erythrocyte SOD in all the groups of experimental animals over the pre exposure values indicates the condition of oxidative stress during heat exposure at exposure I and II in climatic chamber. Bernabucci *et al.* (2002) also reported a significant increase in erythrocyte SOD activity in Holstein cows due to moderate heat stress indicating a condition of oxidative stress in summer transition dairy cows. Oxidative stress resulting from increased production of free radicals and reactive oxygen species, and decrease in antioxidant defense, leads to damage of biological macromolecules and disruption of normal metabolism and physiology (Trevisan *et al.* 2001). These conditions can contribute and/or lead to the onset of health disorders in cattle

(Miller *et al.* 1993).

Catalase (EC 1.11.1.6): The increase in Catalase activity after 3 hr of exposure at both the exposures I and II were 15.00% and 28.47% respectively over the initial values in growing buffaloes. The respective increases in erythrocyte Catalase activity in buffalo heifers were 8.99% and 25.00% and in lactating buffaloes the values were 23.92% and 33.18% after 3 hrs of exposure at I and II conditions respectively (Table 2). The levels of Catalase activity was significantly (P<0.01) higher in all groups of buffaloes after 3 h of exposure at both the exposures over pre exposure values. The magnitude of increase in Catalase activity during exposure II was significantly (P<0.01) higher compared to exposure I in all groups of buffaloes. The analysis of variance of data on Catalase revealed a significant (P<0.05) difference between stages, treatments, hours, and interactions between treatments×hours (Table 3).

Catalase is one of the antioxidant enzyme which catalyzes the degradation of H_2O_2 to water and oxygen (Switala & Loewen 2002). Catalase activity is necessary for heat-shock recovery in animal tissue. The results of the present study are in agreement with those of Kumar *et al.* (2005) and Shibu (2007) who reported a significant increase in Catalase activity of Murrah buffaloes and Karan-Fries cattle due to heat exposures in a climatic chamber. Ozturk and Gummuslu (2004) also reported an increased level of Catalase in rats which were exposed to 40°C for 7 days. Frei (1994) observed higher activity of Catalase and Glutathione peroxidase in the blood during summer condition in dairy cows.

HSP72 mRNA expression in lymphocytes: The relative quantity of HSP72 mRNA in growing buffaloes increased from pre exposure value of 0.69±0.02 to 0.70±0.02, 0.74±0.04 and 0.72±0.02 after 1, 2 and 3 h of exposures respectively during exposure I. During exposure II, the respective increase was 0.72±0.02, 0.89±0.05 and 0.73±0.00 after 1, 2 and 3 h of exposure respectively over the pre exposure value of 0.66±0.09. The increase in relative quantity of HSP72 mRNA was 4.35% and 9.18% after 3 h of exposure over the pre-exposure values at exposure I and exposure II respectively (Table 2). The relative quantity of HSP72 mRNA in buffalo heifers were increased from pre exposure value of 0.54±0.01 to 0.68±0.01, 0.72±0.06 and 0.72±0.02 after 1, 2 and 3 h of exposure respectively during exposure I. During exposure II, the respective increase was 0.75±0.04, 0.86±0.06 and 0.74±0.02 after 1, 2 and 3 h of exposure respectively over the pre exposure value of 0.52±0.01. HSP72 mRNA expression increased significantly (P<0.05) i.e., 33.33 and 40.38% over the pre exposure values at exposure I and II respectively (Table 2). The relative quantity of HSP72 mRNA in lactating buffaloes increased from the pre-exposure value of 0.62±0.02 to 0.73±0.05, 0.72±0.01 after 1 and 2 h of exposure respectively and then declined to 0.63±0.02 after 3 h of exposure during exposure I. During exposure II, similar trend was observed i.e., increased HSP72 mRNA expression from 0.59±0.09 to 0.76±0.04 and 0.74±0.08 after 1 and 2 h of exposure respectively and then declined to 0.65±0.01 after 3 h of exposure over the pre exposure values. Heat exposure increased expression of HSP72 mRNA after 1 h and then decline in both the exposures (Table 2). The HSP72 mRNA expression increased significantly (P<0.05) after 1 and 2 h of exposures in growing and heifers during both the exposures whereas, in lactating buffaloes a declining trend was observed after 1 h of exposure during both the exposures. The magnitude of increase in the expression of HSP72 mRNA was significantly (P<0.05) higher during exposure II compared to exposure I in all groups of buffaloes. Analysis of variance of HSP72 mRNA data revealed a significant (P<0.01) difference between treatment, hours and interactions between treatment×hours (Table 3).

The results of the present study are in general agreement with Mayengbam (2008) who reported a relative range of HSP72 mRNA response from 0.45±0.18 to 1.15±0.18 in Sahiwal and 0.69±0.09 to 1.28±0.31 in Karan-Fries cattle after exposing the animal in climatic chamber at 40°C and 45°C with 50% RH. The pattern of Heat Shock Protein induction under the present study are in accordance to Patir and upadhyay (2007) who indicated a rise in HSP70 concentration after two h of exposure at 45°C and then the concentration declined after 3 and 4 h of exposures in Murrah buffaloes. King *et al.* (2002) also reported an induction of HSP70 at 41°C in mice after 30 min of exposure and concluded that HSP70 performed a significant role to help in the survival of mice during heat stress. Sahin *et al.* (2009) reported increased levels of HSP70 expression and corticosterone in brain and ovary during heat exposure at 34°C for 8 h/day in Japanese quail. The detectable levels of HSP before exposure to climatic chamber conditions during the present study indicate that the natural environmental condition was also stressful to elicit the production of HSP. Kamwanja *et al.* (1994) also reported an induction of heat shock protein after 1 h exposure at 42°C with 2 to 3 fold increases in the intracellular concentrations of HSP70 in the lymphocytes of different cattle breeds. Similarly in present study, there was also a sharp rise of HSP72 mRNA expression at one point of exposure and declined thereafter in lactating buffaloes (Table 2). This may be due to the physiological mechanism of the animals that had increased the expression of HSP72 to cope up with the change in the heat exposure for longer duration. This phenomenon of acquired thermotolerance is transient in nature and depends primarily on the severity of the initial heat stress on the animals. Acute exposure to heat has been shown consistently to induce heat shock response *in vitro* and *in vivo* in heifers (Kennie 2000). Theodorakis *et al*. (1999) also found that thermotolerant cells expressed less HSP72 when subjected to heat shock. The increased expression of HSP72 due to increase in temperature in climatic chamber may be due to the stress that in turn change in intracellular p^H, cyclic AMP, Ca2+, Na+, inositol

triphosphate, protein kinase C and protein phosphatases. HSP72 acts as molecular chaperone for cytoprotection preventing inappropriate protein aggregation and to mediate transport of immature proteins to the target organelles for final packaging, degradation, or repair as reported by Kiang and Tsokos (1998). Due to this mechanism of its unique action, the HSP70 family itself is one of the most studied families of all the HSPs.

In conclusion, under heat stress, free radicals are produced in the body which are neutralized by interacellular antioxidant enzymes produced in the different tissues of the body depending upon their metabolic activity. In present study the levels of erythrocytic SOD and Catalase increased significantly after three h of exposures in climatic chamber. The magnitude of increase in SOD and Catalase was found to be significantly higher at exposure II compared to exposure I. The HSP 72 mRNA expression increased significantly after end of two h of exposures in growing and heifers of Murrah buffaloes during both the exposures where as in lactating buffaloes the HSP 72 mRNA expression increased during first two h of exposures and later on a declining trend was observed at third hour. Based on this study, it can be concluded that exposure of buffaloes to high ambient temperature is the major constraint for production in the tropical and subtropical climatic conditions.

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