



Effect of summer stress and supplementation of vitamin E and selenium on heat shock protein 70 and anti-oxidant status in Hallikar cattle

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ABSTRACT

Present study was conducted to ascertain the influence of supplementation of vitamin E and selenium on heat shock protein 70 (HSP70) and anti-oxidant status in Hallikar cattle during different seasons. Female Hallikar cattle (12) aged 4 to 6 years selected from Ramanagara, Karnataka, India, were divided into control and supplemented groups with 6 animals in each group. Selected animals were exposed to environmental stressors during 3 different seasons (winter, summer and rainy) by allowing them for free grazing. Animals of supplemented group received oral supplementation of vitamin E and selenium, and control group animals did not receive any supplementation. Blood samples collected from each animal at monthly interval were utilized to determine plasma levels of HSP70 and erythrocyte activities of catalase, superoxide dismutase and glutathione peroxidase in hemolysates (10%). Present study showed significant increase in plasma HSP70 levels during summer compared to winter in control and supplemented groups. However, plasma HSP70 levels did not vary significantly between control and supplemented group during different seasons. Activities of catalase, superoxide dismutase and glutathione peroxidase enzymes were also significantly higher during summer compared to other seasons in both control and supplemented group. However, activities of these enzymes reduced significantly in supplemented group compared to control group animals. From the study, it was concluded that significantly lowered antioxidant enzyme activities in supplemented group indicate beneficial effects of supplementation of vitamin E and selenium during summer.

Keywords: Anti-oxidant status, Cattle, Hallikar, Heat shock protein 70, Selenium, Summer stress, Vitamin E

Heat stress can be defined as the point where the animal cannot dissipate adequate quantity of heat to maintain body thermal balance. Heat stress is the most important climatic stress, which adversely affects the livestock and has become major issue in the changing climate scenario. At the cellular level, individuals respond to stresses by producing a set of proteins called heat shock proteins (HSPs) which play vital role in cryoprotection and protein homeostasis (Guerriero and Raynes 1990). There is a strong relationship between HSP70 concentration and ambient temperature (Gaughan *et al.* 2013) and HSP70 is frequently used as a biomarker of cellular stress. Levels of HSP70 are indicative of magnitude and duration of the thermal stress in animals (Rhoads *et al.* 2013). Understanding biological mechanisms of heat stress is essential for designing the future stress mitigating strategies to improve animal performance. Experiments on farmer owned animals in their own premises at rural conditions are known to give more realistic results than on-station experiments. The present study was undertaken to determine the influence of dietary supplementation of vitamin E and selenium on heat shock protein 70 and anti-oxidative status in Hallikar cattle during summer stress.

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MATERIALS AND METHODS

Location and duration of the study: Present study was conducted in Magadi, Karnataka, India, a semi-arid region with an average annual rainfall of 795 mm and maximum temperature recorded over the years (past 20 years) was around 38°C during summer and the minimum was around 12°C in winter. Study period included winter months (January and February 2014), summer months (April and May 2014) and rainy months (July and August 2014). Daily minimum and maximum temperature (°C) and daily minimum and maximum relative humidity (%) were obtained for the study period (January to August 2014) from internet sources (<http://www.accuweather.com>).

Assessment of heat stress: Utilizing the recorded monthly meteorological data on temperature and relative humidity, the temperature humidity index (THI) for the different months during the entire study period was calculated using the formula as mentioned by Dikmen and Hansen (2009).

$$THI = (1.8 \times T_{db} + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times T_{db} - 26.8)]$$

where T_{db} , average dry bulb temperature (°C); RH, Average relative humidity (%).

Animal selection: Apparently healthy female Hallikar cattle (12) free from physical or anatomical abnormalities

and aged 4 to 6 years were randomly selected from farmers of Madabal village of Ramanagara District, Karnataka, India, for the present study. All the study animals were maintained in semi-intensive housing system with uniform feeding and managerial practices in the farmers' premises. Selected animals were placed in control and supplemented group, with six animals in each group. Animals of both the groups were fed with 8–10 kg/day of dry fodder and 4–5 kg/day green fodder with *ad lib.* water during entire period of the study. All the animals were exposed to environmental stressors for three different periods of the year, viz. winter months, summer months and rainy months by allowing them for free grazing for 7 h daily from 10:00 AM to 05:00 PM. Though some of the selected animals exhibited the estrous during the study period, they were not inseminated until the completion of the study so as to maintain the uniformity among the study animals.

Supplementation of vitamin E and selenium: Vitamin E (D-alpha tocopherol acetate) and selenium (sodium selenite) in the form of powder with 50% and 45% purity, respectively, were procured from Provimi Animal Nutrition India Pvt. Ltd., Bengaluru, India. Oral dosages of the Vitamin E and selenium were fixed as detailed below. Animals of control group received no supplementation, while supplemented group received vitamin E (D-alpha tocopherol acetate) with oral dose @ 1,000 IU/day/animal (Chandra *et al.* 2013) and selenium (sodium selenite) with oral dose @ 0.3 ppm/kg dry matter intake (NRC 2001) in addition to regular diet for the entire period of the study.

Sample collection and processing: Blood samples from all animals were collected during morning hours (08:00 to 09:00 AM) in all the three study periods on the last day of every month in the respective period. From each animal, 5 ml of blood sample was collected in heparinized vacutainer and samples were transported to the laboratory in refrigerated temperature within 2 h after collection for further processing. Blood plasma was separated from heparinized blood by centrifuging at $700 \times g$ for 15 min. The plasma samples obtained were stored at -80°C until they were used for the analysis of heat shock protein 70 (HSP70). The erythrocyte pack obtained after the separation of plasma was utilized immediately to prepare the hemolysate (10%).

Preparation of hemolysate for antioxidant assay: Packed red blood cells were mixed with equal volume of phosphate buffer saline (pH 7.4) and centrifuged at $700 \times g$ for 15 min. The supernatant was removed and the cellular pack was mixed with phosphate buffer saline (pH 7.4) and centrifuged. This procedure was repeated 3 times. Finally 100 ml of washed red cell volume was mixed with 900 ml chilled distilled water to obtain 10% hemolysate. The obtained hemolysates were centrifuged at $10,000 \times g$ for 5 min to obtain membrane free hemolysate which was later stored at -80°C in different aliquots until they were analyzed for the activities of various antioxidant enzymes, viz. erythrocyte catalase (CAT), erythrocyte superoxide dismutase (SOD)

and erythrocyte glutathione peroxidase (GPx).

Determination of haemoglobin: Part of the hemolysate (10%) prepared for the determination of antioxidant enzyme activities was immediately utilized for the estimation of haemoglobin using cyanomethemoglobin method (Sharma *et al.* 2011).

Determination of plasma HSP70: The concentration of heat shock protein 70 (HSP70) in the blood plasma sample was determined by Bovine HSP70 ELISA reagent kit manufactured by CUSABIO, China, as per the protocol provided by the manufacturer.

Determination of erythrocyte antioxidant enzyme activity: The activity of erythrocyte catalase in the hemolysate was determined using the method described by Caliborne (1985). Activity of superoxide dismutase (SOD) in the hemolysate was determined using the method described by Marklund and Marklund (1974) and activity of glutathione peroxidase was determined by the method described by Rotruck *et al.* (1973).

Statistical analysis: All the data were analyzed using computerized statistical software programme, GraphPad Prism version 5.01 (2007) by applying two-way ANOVA with Bonferroni post ANOVA mean comparison test. The significance was determined at probability levels of 95% ($P < 0.05$).

RESULTS AND DISCUSSION

Temperature humidity index (THI): The mean THIs were 69.97 ± 1.14 , 79.52 ± 0.12 and 75.29 ± 1.09 during the winter, summer and rainy months, respectively, indicating the significant stress during the summer months (April and May).

The mean plasma concentrations of HSP70, mean erythrocyte activities of catalase, superoxide dismutase and glutathione peroxidase enzymes are presented in Table 1.

Plasma HSP70: The mean HSP70 levels were significantly ($P < 0.05$) higher in control and supplemented group animals during summer as compared to winter. Significantly higher HSP70 levels during summer were also reported by Dangi *et al.* (2012) in goats, Gaughan *et al.* (2013) in crossbred cattle, Deb *et al.* (2014) in Sahiwal and Frieswal cattle and Kumar *et al.* (2018) in Hariana and Sahiwal cattle. Heat tolerance at cellular level is directly proportional to the ability of cell to maintain elevated levels of HSPs (Roy and Collier 2012), higher levels of HSP70 during summer indicated higher expression of HSP70 in animals exposed to summer temperature in order to provide better cellular protection against the deleterious effects of thermal stress. Higher levels of HSP70 might also improve the cell survivability by reducing the accumulation of abnormal or damaged proteins in the cells and thus reducing the heat induced cellular apoptosis (Gabai *et al.* 1998). Significantly higher levels of plasma HSP70 during summer season could also be due to higher levels of cortisol which is known to release preformed HSP70 (Behl *et al.* 2010). The non-significant variation in plasma HSP70 levels between control and supplemented groups indicated that

Table 1. Mean±SE values of plasma HSP70 (ng/mL), erythrocyte catalase (mmol of H₂O₂/min/g of Hb), superoxide dismutase (units/g of Hb) and glutathione peroxidase (units/g of Hb) activities in Hallikar cattle during different seasons (n=6)

Season	Plasma HSP70		Catalase		Superoxide dismutase (SOD)		Glutathione peroxidase (GPx)	
	Control group	Supplemented group	Control group	Supplemented group	Control group	Supplemented group	Control group	Supplemented group
Winter	2.39± 0.09 ^{aA}	2.43± 0.18 ^{aA}	157.66± 3.07 ^{aA}	134.15± 5.48 ^{aB}	296.00± 4.49 ^{aA}	157.26± 4.76 ^{aB}	3031.25± 30.42 ^{bA}	2689.23± 83.30 ^{aB}
Summer	3.08± 0.14 ^{bA}	3.52± 0.20 ^{bA}	188.79± 2.75 ^{bA}	163.13± 2.08 ^{cB}	358.58± 1.43 ^{bA}	195.62± 4.89 ^{bB}	3368.10± 24.0 ^{cA}	2971.09± 44.92 ^{bB}
Rainy	2.70± 0.08 ^{abA}	2.74± 0.19 ^{aA}	161.18± 3.74 ^{aA}	150.45± 3.00 ^{bA}	299.28± 9.70 ^{aA}	187.58± 5.02 ^{bB}	2845.98± 62.23 ^{aA}	2596.98± 37.45 ^{aB}

The mean±SE values with different superscripts within a column (a, b and c) and within a row (A and B) differ significantly (P<0.05).

the vitamin E and selenium supplementation did not influence plasma HSP70 levels in Hallikar cattle.

Catalase activity: Erythrocyte catalase activity in control and supplemented groups was significantly higher (P<0.05) during summer as compared to winter and rainy seasons. Similar findings were also reported by Kumar *et al.* (2007) in cattle and buffaloes and Ganaie *et al.* (2013) and Lallawmkimi *et al.* (2013) in Murrah buffaloes. Higher catalase activity during summer could be attributed to increased oxidative stress leading to higher generation of free radicals and stimulation of endogenous antioxidant defence system to quench these reactive oxygen species. As catalase is known to catalyse dismutation of hydrogen peroxide into water and oxygen, higher catalase activity during summer could help in quenching the H₂O₂ produced during summer due to increased activity of superoxide dismutase. Significant decline (P<0.05) in catalase activity of supplemented group as compared to control group animals during summer and winter seasons indicated the beneficial effects of antioxidant supplementation during these seasons. Significant reduction in catalase activity in supplemented group was also reported by Kumar *et al.* (2011) and Lallawmkimi *et al.* (2013) in buffaloes supplemented with ascorbate along with salt and vitamin E, respectively. Vitamin E acts as a primary antioxidant of cell membrane and its supplementation through diet could reduce reactive oxygen species and prevent the lipid peroxidation of biological membrane thus resulting in reduced oxidative damage to the cells (Sordillo 2013).

Superoxide dismutase activity: Significantly higher (P<0.05) superoxide dismutase (SOD) activity in control group recorded during summer season as compared to winter and rainy seasons was in accordance with findings of Kumar *et al.* (2011) in buffaloes, Chigerwe *et al.* (2013) in neonatal dairy calves, Yattoo *et al.* (2014) in lactating and non-lactating cattle and Lakhani *et al.* (2018) in Murrah buffaloes. Being considered as first line of defence against the pro-oxidants, higher SOD activity during summer might be aimed at preventing oxidative injury to cells. Higher SOD activity could be due to heat stress induced generation of the free radicals especially superoxide anion and could

also be due to auto-oxidation of haemoglobin resulting in generation of superoxide anion in erythrocytes. In conjugation with catalase and glutathione peroxidase, superoxide dismutase scavenges both intracellular and extracellular superoxide anion radicals and prevents lipid peroxidation. Significant (P<0.05) reduction in SOD activity of supplemented group during all the seasons was in agreement with reports of Chandra and Aggarwal (2009) in cows, Sunil Kumar *et al.* (2011) in buffaloes and Lallawmkimi *et al.* (2013) in buffaloes, who reported significantly decreased SOD activity in groups supplemented with DL- α -tocopherol, electrolytes along with ascorbic acid and zinc and vitamin E, respectively. Significant reduction in erythrocyte SOD activity in supplemented group observed in the present study could be attributed to sparing of endogenous enzymatic antioxidants by supplemented non enzymatic antioxidants during oxidative stress indicating the beneficial effects of antioxidants like vitamin E and selenium during the thermal stress.

Glutathione peroxidase activity: The glutathione peroxidase activity in both the groups was significantly higher (P<0.05) during summer season as compared to winter and rainy seasons and the activity was significantly reduced in the supplemented group as compared to control group during all the seasons. Higher GPx activity might be due to increased generation of hydrogen peroxide by enhanced activity of SOD during summer as observed in the present study. Similar findings were also recorded by Bernabucci *et al.* (2002) in Holstein cows and Chigerwe *et al.* (2013) in neonatal dairy calves. The dismutation of super oxide by enhanced SOD activity during summer results in increased production of hydrogen peroxide and protection from this ROS would only be conferred by coordinated increase in the activity of catalase and glutathione peroxidase (Chandra and Aggarwal 2009). Significant reduction in erythrocyte GPx activity in the supplemented group compared to control group could be due scavenging of free radicals by vitamin E and selenium resulting in reduced oxidative stress.

From the present study, it was concluded that the enhanced

expression of HSP70 and subsequent increase in its concentration in the plasma of Hallikar cattle during summer season could confer the ability to maintain cellular integrity during thermal stress. Significant reduction in antioxidant enzyme activities in vitamin E and selenium supplemented animals compared to control animals during summer season indicated reduced oxidative stress in these animals. However, antioxidant supplementation did not influence the plasma HSP70 levels in heat stressed Hallikar cattle.

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