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Comparative estimation of total antioxidant capacity in plasma of crossbred calves under controlled climatic conditions

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Abstract
Heat stress in livestock is the global challenge affecting their overall production and productivity. Cattle initiate various adaptive mechanisms to maintain thermal homeostasis. Increased oxidative stress in cattle disrupts the redox homeostasis making them more vulnerable to cellular damage. The Total Antioxidant Capacity (TAC) represents the ability of animal's endogenous antioxidant defense mechanism to neutralise the reactive oxygen species produced during heat stress. The present study was conducted with the objective of comparing the TAC of crossbred calves under thermo-neutral zone (TNZ) and acute heat stress conditions in a climate-controlled chamber. The TAC values were measured as ferric reducing ability of plasma (FRAP) by the method of Benzie and Strain (1996). The results of the study showed that FRAP values were significantly increased on different days of heat exposure when compared to TNZ. The present finding concluded that TAC of crossbred (Holstein Friesian × Sahiwal) calves increased during acute heat stress to prevent from cellular damage due to oxidative stress and the values remained constant throughout the thermo-neutral condition, indicating the absence of oxidative stress under TNZ.

Keywords: Heat stress, thermo-neutral zone, crossbred calves, total antioxidant capacity, FRAP, climatic chamber

1. Introduction
Heat stress (HS) is the major global challenge to the livestock affecting their overall well-being, production and productivity. Cattle maintains their thermal equilibrium by initiating various thermo-regulatory and adaptive mechanisms which promote their welfare and survival under stressful conditions (Indu *et al*., 2015) [10]. Cattle possess a thermo-neutral zone (TNZ) or comfort zone within which all their physiological and biological functions will be normal. If the temperature of the animal exceeds the upper critical temperature (UCT) of TNZ, the animal experience HS (Bagath *et al*., 2019) [3]. Cattle are very sensitive to UCT and it has been reported that even a 1 °C rise in temperature from the UCT can affect the production of livestock (McManus *et al*., 2009) [12]. The antioxidant response is one of the primary defense responses in cattle to heat-stressed conditions. The ferric reducing ability of plasma (FRAP) assay is the direct method that reflect the total antioxidant capacity (TAC) of plasma. Heat stress in cattle often result in oxidative stress and disrupt cellular homeostasis (Ganaie *et al*., 2013) [8]. It leads to the production of reactive oxygen species (ROS) such as superoxide, peroxide, hydroxyl radical, singlet oxygen etc. Their overproduction or decreased antioxidant defense mechanism will damage the nucleic acids, proteins and results in lipid peroxidation and disruption of normal cellular metabolism. Antioxidant enzymes provide protection from ROS produced during HS (Gupta *et al*., 2021) [9]. They scavenge both intracellular and extracellular superoxides and inhibit lipid peroxidation of plasma membrane (Zhang, *et al*., 2017) [15]. The endogenous antioxidant capacity comprises of three major groups of antioxidants which contributes to the antioxidant capacity of plasma. The first group includes enzymatic antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT).

They are the chief intracellular antioxidant defense system against superoxides and hydroperoxides. The second group includes protein antioxidants in the intracellular fluids such as sulfhydryl groups of albumin, cysteine and homocysteine. The third group includes water-soluble vitamin C, glutathione and the lipid soluble vitamin E. These antioxidants quench the free radicals and counteract the oxidative stress, contributing to higher FRAP value. Thio-barbituric acid reactive substance (TBARS) is also one of the oxidative stress markers in the plasma. The upregulation of mRNA expressions of antioxidant genes such as GPx, SOD and CAT leads to higher enzyme activity, enhancing the capacity of the cells to detoxify ROS and maintain redox balance (Akbarian *et al*., 2014) [1]. In view of this, the present study was conducted with the objective of comparing the TAC of plasma in crossbred (Holstein Friesian × Sahiwal) calves under TNZ and acute heat stress in a climate-controlled chamber.

2. Materials and Methods

2.1 Ethical approval and experimental layout

The experiment was conducted at Climate Controlled Research Complex (CCRC) of Centre for Animal Adaptation to Environment and Climate Change Studies (CAADECCS) of Kerala Veterinary and Animal Sciences University following the approval of Committee for the Control and Supervision of Experimentation on Animals (CCSEA), New Delhi. Six healthy crossbred (Holstein Friesian × Sahiwal) female calves of eight to twelve months of age were selected randomly from University Livestock Farm, Mannuthy. Same six animals (Figure 1) were distributed in two experimental groups: thermo-neutral zone (TNZ) or control group; acute heat stress or treatment group. The research was initiated with acclimatisation period of 10 days each in the animal holding facility and in the climatic chamber for adaptation of animals to the novel environmental conditions. It was followed by experimental period of 10 days housed under TNZ and 10 days for acute HS study. In TNZ, animals were maintained at a temperature of 27 °C and relative humidity of 45-55 percent. In acute HS study, maximum temperature of 40 °C and relative humidity of 55-65 percent was simulated for three hours a day for 10 days. After the experiment, the animals were re-shifted to the farm for rehabilitation and reuse as recommended by CCSEA.

Fig 1: Experimental crossbred (Holstein Friesian × Sahiwal) calves kept in the climatic chamber of Climate controlled research complex (CCRC), CAADECCS, KVASU

2.2 Collection of blood samples

Venous blood samples were collected in lithium heparinised vacutainers in both the experimental groups on days zero, one, five and ten of the experiment and immediately centrifuged at 2700×g at 4 °C for 15 minutes to separate plasma. Plasma samples were stored at -80 °C until complete preparation for analysis. The estimation was done instantaneously on the same day after the separation of plasma for higher accuracy of antioxidant capacity.

2.3 Estimation of total antioxidant capacity

The ferric reducing ability of plasma (FRAP) assay by Benzie and Strain (1996) [4] was used as a direct method for estimating the total antioxidant capacity of plasma samples. At low pH, ferric 2, 4, 6-tripyridyl-s-triazine [Fe (III)-TPTZ] complex gets reduced to ferrous 2, 4, 6-tripyridyl-s-triazine [Fe (II)-TPTZ] complex, which gives an intense blue colour, can be monitored by measuring the change in absorption at 593 nm. Working FRAP reagent was prepared by using 300 mM acetate buffer, pH 3.6 (3.1 g sodium acetate trihydrate, plus 16 mL glacial acid); 10 m*M* TPTZ [2, 4, 6-tris (2-pyridyl)-s-triazine], in 40 m*M* HCl; and 20 m*M* $FeCl_3 \bullet 6H_2O$ in the ratio of 10:1:1. Plasma sample (100 µL) was mixed with 3 mL of FRAP reagent and kept for 10 minutes. Then the sample was centrifuged at 10,000 rpm for 5 minutes and supernatant solution was taken. The absorbance of the supernatant solution was monitored at wavelength 593 nm with Perkin Elmer's LAMBDA UV-Vis spectrophotometer in the Central Instrumentation Laboratory (CIL). Aqueous solution of Ascorbic acid (1000 µ*M*) was used as standard. The change in absorbance was translated into FRAP value (µ*M*) by relating ΔA_{593} of test sample with that of standard solution of known FRAP value using formula.

Calculation of FRAP Value

$$\frac{0 - 5 \text{ min } \Delta A_{593} \text{ test sample}}{0 - 5 \text{ min } \Delta A_{593} \text{ standard}} \text{ x FRAP value of standard}$$

2.4 Statistical analysis

The results were expressed as Mean ± Standard Error. The statistical analysis of the calculated data was done by linear mixed model analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS) version 24.0 software.

3. Results and Discussion

The overall antioxidant capacity of plasma was significantly increased ($p<0.001$) in the crossbred calves (Holstein Friesian × Sahiwal) exposed to acute heat stress when compared to calves kept under TNZ (Table 01). The mean FRAP values differ significantly between day zero and 10 of the calves under TNZ. Whereas in acute heat stressed group, FRAP value was gradually increased from day one of heat exposure to day ten. The highest mean FRAP value of 3188.45±95.15 µ*M* was recorded on day ten of heat exposure and the lowest value of 1221.89±134.89 µ*M* on day zero or before the heat exposure. Hence the TAC was increasing as the treatment days advanced indicating the gradual increase in oxidative stress throughout the acute HS period (Figure 2). But the FRAP value remained constant on different days in calves kept in thermo-neutral condition.

Table 1: Total antioxidant capacity (TAC) measured as ferric reducing ability of plasma (µ*M*) in crossbred (Holstein Friesian × Sahiwal) calves under thermo-neutral zone and acute heat stressed conditions (Mean ± SE, N=6)

Days group	Day 0	Day 1	Day 5	Day 10	Overall mean
Thermo-neutral zone	971.145^{aA} ±25.85	1028.83^{aA} ±28.07	1043.81^{aA} ±54.76	1030.86^{aA} ±72.57	1018.67^{A} ±24.65
Acute heat stress	1221.89^{aA} ±134.89	1764.92^{aB} ±212.1	2675.79^{bB} ±237.06	3188.45^{bB} ±95.15	2212.77^{B} ±89.59
P-value	0.123^{ns}	<0.001	<0.001	<0.001	<0.001

Means bearing different superscript within a row (a-b) and columns (A-B) differ significantly ($P<0.05$), ns – non- significant at 0.05 level

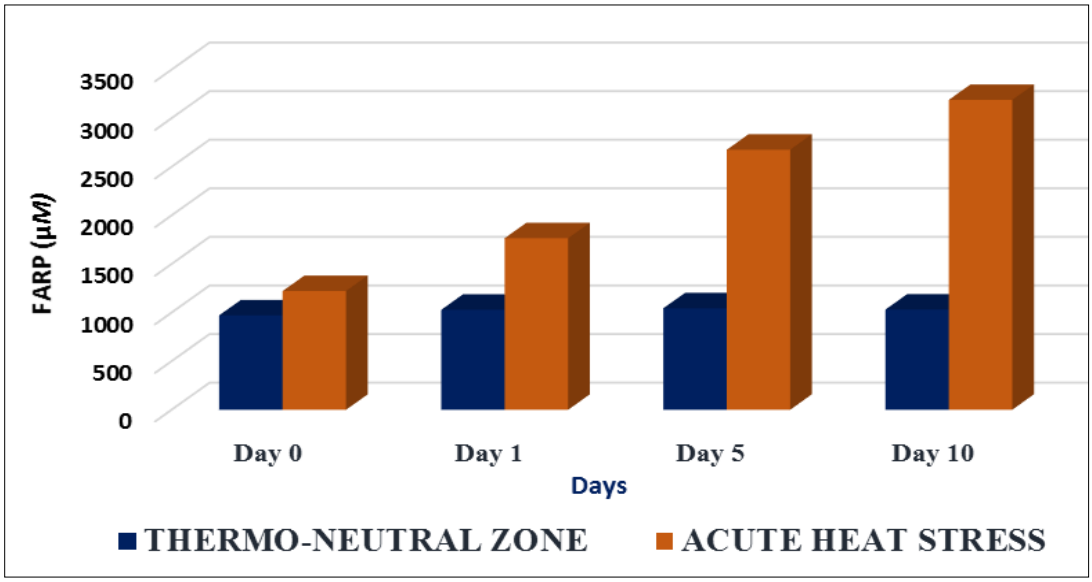

Fig 2: Ferric reducing ability of plasma (µ*M*) in crossbred (Holstein Friesian × Sahiwal) calves under thermo-neutral zone and acute heat stress

The present findings of increased FRAP value during HS is in accordance with the findings of Almoosavi *et al*. (2020) [2] who reported that FRAP value was significantly increased ($p<0.001$) in the heat-stressed cows when compared to the cows under cooled conditions. Higher TAC value during HS was also reported by Chaiyabutr *et al*. (2011) [6], Chaudhary *et al*. (2015) [7], Tejaswi *et al*. (2020) [14] and Cecchini and Fazio (2020) [5] further validating the present findings.

Higher TAC value during HS was attributed to the production of antioxidant enzymes such as SOD, catalase and GPx, and non-enzymatic antioxidant such as vitamin E that might play a role in quenching the free radicals (Chaiyabutr *et al*., 2011) [6]. Another reason for increased FRAP value during HS might be that the enzymatic antioxidants could already neutralise the ROS produced as a result of oxidative stress, thereby increased the overall antioxidant capacity of plasma. The increased FRAP value might also be due to upregulation of heat shock protein-70 (*HSP-70*) gene expression during HS which co-works with antioxidants in maintaining the cellular integrity (Khan *et al*., 2020) [11]. According to Akbarian *et al*. (2014) [1] and Rimoldi *et al*. (2015) [13], upregulation of antioxidant genes such as GPx, SOD (SOD 1 and SOD 2) and CAT leads to higher enzyme activity, increasing the TAC of plasma. The increased FRAP value could be considered as an indicator of adaptive antioxidant defence mechanism in crossbred calves to HS. The constant FRAP value in the control group of the present study indicates the absence of oxidative stress in crossbred (Holstein Friesian × Sahiwal) calves kept under TNZ.

4. Conclusion

This study highlighted the normal cellular functioning of crossbred (Holstein Friesian × Sahiwal) calves under thermo-neutral zone. The present findings also elucidated the antioxidant defense mechanism adapted by crossbred (Holstein Friesian × Sahiwal) calves when exposed to a maximum temperature of 40ºC and relative humidity of 55-65 percent, to counteract the oxidative stress due to environmental challenges. Hence it is suggested to maintain cattle under their TNZ for their normal biological functions and welfare.

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