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***Invitro* Effect of Zinc Treatment on the Antioxidant Status of Heat Stressed Peripheral Blood Mononuclear Cells of Periparturient Sahiwal and Karan Fries Cows - A Comparative Study**

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ABSTRACT

The current study was undertaken to evaluate the *invitro* effect of zinc treatment on the Superoxide Dismutase (SOD) and Catalase status in the heat stressed Peripheral Blood Mononuclear Cells (PBMC) of periparturient indigenous and crossbred cows. Twelve pregnant cows, six each from Sahiwal and Karan Fries in their second parity were selected for the experiment. Blood samples were collected at -21, 0 and +21 days with respect to calving from each animal. The 48 hour long cultured PBMC were exposed to three levels of treatment viz., 37°C, 42°C to induce thermal stress and 42°C+Zinc to minimize the effect of high temperature. SOD and Catalase showed a significant ($P<0.05$) difference between the two breeds, more concentration being found in Karan Fries. While comparing the days, the concentration of both SOD and Catalase was found to be more on the day of calving though non-significantly in case of SOD but the difference was significant ($P<0.05$) vis-à-vis Catalase. Zinc treatment caused a decreased production of both SOD and Catalase which were otherwise increased due to thermal stress. The antioxidant concentration was highest due to thermal stress in Karan Fries on the day of calving. The oxidative stress supervenes during peripartum and heat stress which could be alleviated by zinc treatment.

Keywords: PBMC, Heat stress, Zinc, SOD, Catalase, Periparturient

The problem of thermal stress in dairy cattle has lately received more attention due to expected increase in temperature by global warming (Hansen, 2004; Hoffmann, 2010). Heat stress leads to noticeable loss to animal production systems in tropics (Hansen, 2009). The periparturient period is characterized by notable metabolic and endocrine adjustments that cows experience from late gestation to the early lactation (DeFrain *et al.*, 2005). Transition period is to a greater extent critical for health and overall performance of dairy cows (Castillo *et al.*, 2005). Dairy cattle are more vulnerable to a variety of metabolic and infectious diseases during the periparturient period compared to peak lactation (Sharma *et al.*, 2011). There are increasing evidences that oxidative stress is an imminent danger to transition period and elevated levels may lead to calving-related complications in

both man and animals (Orhan *et al.*, 2003). Zinc plays a critical role in anti-oxidant defense as an immanent part of the essential enzyme superoxide dismutase (SOD) (Underwood, 1999; National Research Council, 2001). Zinc is a strong mediator of host resistance to infection. Even marginal zinc deficiency considerably suppressed peripheral blood lymphoid cell concentrations in mice and humans (Prasad, 1998). Plasma zinc concentrations get reduced in dairy cows at calving and return to normal values within 3 days (Goff and Stable, 1990). As per NRC (2001) guidelines, the dairy cow requires more zinc (63 mg/kg DM) as compared to beef cattle (40 mg/kg DM). The mean plasma Zn level of Karan Fries cows was 1.83 ± 0.04 ppm 60 days prepartum and on the day of calving, it decreased to 1.27 ± 0.03 ppm (Maurya *et al.*, 2014). Body has antioxidants that exist as enzymes (SOD, Catalase and



Glutathione peroxidase) and non-enzymes (Vitamin C, E and A, glutathione pyruvate, *etc*). There is a pronounced activity of SOD during the last three weeks of pregnancy, and after calving, the SOD activity declined (Bernabucci *et al.*, 2005). In Holstein X Sahiwal crossbred dairy cows, it was found that Catalase increased from 42.52 ± 6.98 to 48.33 ± 6.55 $\mu\text{moles of H}_2\text{O}_2$ decomposed/min/mg Hb during advanced pregnancy to early lactation whereas, SOD decreased from 6.99 ± 0.45 to 6.37 ± 0.72 units/mgHb during the same time period (Sharma *et al.*, 2011). It was reported that the plasma SOD concentration in Karan Fries cows declined around parturition (Aggarwal *et al.*, 2013). Erythrocyte TBARS (Thiobarbituric acid reactive substances), SOD (Superoxide Dismutase), and SH (Intracellular thiols) were higher (P ranging from < 0.05 to < 0.01) in Summer cows than in their Spring counterparts and these parameters increased as the pregnancy advanced (Bernabucci *et al.*, 2002). SOD along with Catalase and glutathione peroxidase (GPx) remove both intracellular and extracellular superoxide radicals and prevents lipid peroxidation (Aggarwal and Prabhakaran, 2005). Exposure to superoxide anion in the presence of the free radical scavenging enzymes, superoxide dismutase, and catalase enhanced cell survival and prevented HSP induction in human (Omar and Pappolla, 2005). Dermal fibroblasts of Tharparkar were seen to be more heat tolerant than crossbred Karan-Fries cattle (Singh *et al.*, 2014). Taking previous studies with their conflicting results into account, the objective of this study was to analyse the antioxidant effect of zinc on heat stressed PBMC in transition Sahiwal and Karan Fries cows.

MATERIALS AND METHODS

The current study was conducted on Sahiwal and Karan Fries (HF \times Tharparkar) dairy cows maintained at Livestock Research Centre (LRC), National Dairy Research Institute (NDRI), Karnal. Two groups of healthy Sahiwal and Karan Fries cows in their transition state in the age group of 4 years were selected for the experiment. Each group contained six animals. The experiment was approved by the Institutional Animal Ethics Committee constituted as per the article number 13 of the CPCSEA rules, laid down by the Government of India.

Feeding and Maintenance of animals

The experimental animals were maintained as per standard practices followed at the LRC, NDRI, Karnal for pregnant animals. All the cows were fed on a ration consisting of concentrate mixture and roughages (berseem, maize or jowar fodder as per the availability in the farm). Throughout the experimental period, animals were given concentrate mixture in the morning @ 1 kg/cow/day, then 2 kg/cow/day 15 days prior to calving. After calving, the cows were given concentrate mixture @ 1 kg/2.5kg of milk produced. Fresh tap water was available throughout the day to all the animals. Animals were maintained in an open area having the brick floor and an asbestos roof. The animals were free to move inside the controlled open area.

Blood collection and PBMC isolation

Blood samples were collected 21 days prepartum, on the day of calving and 21 days postpartum. 10 ml blood was drawn aseptically from jugular vein in sterile Potassium-EDTA coated vacutainer with minimum disturbance to the animal. PBMC were isolated from whole blood by density gradient centrifugation using Histopaque® 1077. The harvested cells were washed with Dulbecco's Phosphate Buffer Saline (DPBS) and then resuspended in 3-4 ml RPMI-1640 medium supplemented with antibiotics and 10% FBS and centrifuged @ 1500 rpm for 10 minutes. The cells were then ready for viability test, enumeration and finally culture.

Viability test, Enumeration and PBMC culture

Trypan blue dye exclusion method was used to determine the proportion of viable cells in the separated PBMC. This method is based on the principle that the dead cells take up the dye, hence they appear blue whereas, live cells appear colorless as they are not stained. For finding out the proportion of live and dead cells in the PBMC cell suspension, 50 μl aliquot of the homogenous suspension was mixed with an equal volume of 0.04% Trypan blue solution (w/v). Neubauer improved double ruling (Haemocytometer) chamber was charged with 10 μl of the above mixture. This was done by touching the edge of cover slip of the Haemocytometer with pipette tip, allowing chambers to get filled by capillary action. The number of colorless viable cells and blue dead cells were counted in the four corners within 10 min of charging the

Table 1: Mean ± SE of Superoxide Dismutase (ng/ml) in Culture supernatant in control and treatment groups during transition period.

BREED	DAYS	LEVEL OF EXPOSURE		
		37 °C (Control)	42 °C (T ₁)	42 °C+Zn (T ₂)
Sahiwal	-21	182.50 ^{Ba} ±10.86	207.52 ^{Aa} ±12.85	183.77 ^{ABa} ±9.68
	0	195.73 ^{BCa} ±18.55	215.05 ^{Aa} ±26.92	207.67 ^{Ba} ±21.68
	21	142.97 ^{Aa} ±15.97	203.40 ^{Ab} ±33.44	164.18 ^{Aa} ±46.01
KaranFries	-21	251.34 ^{DEa} ±16.50	268.03 ^{BCa} ±21.54	243.44 ^{Ca} ±14.49
	0	272.45 ^{Ea} ±12.77	291.19 ^{Ca} ±10.30	278.60 ^{Da} ±12.15
	21	218.76 ^{CDa} ±9.66	257.52 ^{Bb} ±11.84	213.91 ^{BCa} ±32.89

Values (mean ± SE) with different superscript small letters in a row and capital letters in a column differ significantly (P<0.05)

The values of mean ± SE are observations on six animals

Table 2: Mean ± SE of Catalase (IU/ml) in Culture supernatant in control and treatment groups during transition period.

BREED	DAYS	LEVEL OF EXPOSURE		
		37 °C (Control)	42 °C (T ₁)	42 °C+Zn (T ₂)
Sahiwal	-21	25.90 ^{Aa} ±2.96	28.89 ^{Aa} ±2.65	26.28 ^{Aa} ±2.66
	0	39.08 ^{DEab} ±1.25	41.77 ^{Ca} ±1.86	38.17 ^{Cb} ±1.38
	21	30.56 ^{Ba} ±1.73	32.38 ^{Aa} ±1.71	29.03 ^{Aa} ±2.16
Karan Fries	-21	37.72 ^{CDa} ±3.53	38.89 ^{BCa} ±3.08	35.85 ^{BCa} ±2.91
	0	42.11 ^{Ea} ±3.22	45.59 ^{Da} ±3.28	42.34 ^{Da} ±3.35
	21	33.47 ^{Ba} ±2.70	36.94 ^{Ba} ±2.50	34.49 ^{Ba} ±1.85

Values (mean ± SE) with different superscript small letters in a row and capital letters in a column differ significantly (P<0.05)

The values of mean ± SE are observations on six animals

Haemocytometer. With the cover slip in place, each corner of the Haemocytometer represents a total volume of 0.1 mm³ or 10⁻⁴ cm³. As 1 cm³ is equivalent to approximately 1 ml, the cell concentration per ml was obtained as follows:

Total cells per ml = average count (viable + dead cells per square) × 2 × 10⁴

Where 2 represents the dilution factor

Viable cells per ml = average count of viable cells per square × 2 × 10⁴

Total viable cells = concentration of viable cells per ml × the original volume of suspension

$$\text{Cell viability (\%)} = \frac{\text{Total viable cells}}{(\text{Total viable cells} + \text{Total dead cells})} \times 100$$

The cell viability in different experiments was found to be greater than 90% within 4 hours of lymphocyte processing and declined gradually afterwards.

The Peripheral Blood Mononuclear Cell (PBMC) suspension was adjusted to 1×10^6 live PBMC per ml by culture media (RPMI-1640) containing 10% FBS. The cells were dispensed in 75 cm² culture flasks with concentration of 1×10^6 live PBMC per ml. 5 ml of media was dispensed into the flasks. Phytohaemagglutinin (PHA-P) at the concentration of 5 µg/ml was used as mitogen to provide maximal stimulation to bovine PBMC. The cells were allowed to proliferate with PHA-P for 48 hours. The culture flasks without zinc and one containing zinc (0.01mM) were incubated at 37°C in a humidified CO₂ incubator (5% CO₂ and 95% air). After 48 hours, the culture flasks were exposed to three different conditions *viz*;

1. 37°C (acts as control)
2. 42°C (acts as Treatment 1)
3. Flask containing Zinc exposed to 42°C (acts as Treatment 2).

The Culture flasks were subjected to the above mentioned conditions for 3 hours and then brought to the basal temperature (37°C).

Estimation of SOD and Catalase from culture supernatant

Cell supernatant/media was taken into sterile polypropylene centrifuge tubes (15 ml) and was centrifuged @ 3000 rpm for 15 minutes to remove the debris. Samples were stored in the eppendorf tubes at -20°C till they were analyzed for SOD and Catalase.

Cu/Zn-SOD in culture supernatant was determined by “Bovine Cu/Zn-Superoxide Dismutase (Cu/Zn-SOD) ELISA Kit” (catalogue No. CSB-E14090B) from Cusabio Biotech Co.,Ltd. The minimum detectable dose of Bovine Cu/Zn-SOD was typically less than 3.9 ng/ml. The detection range was 15.6-1000 pg/ml.

Catalase in culture supernatant was determined by “Bovine Catalase (CAT) ELISA Kit” (catalogue No. CSB-

E17594B) from Cusabio Biotech Co., Ltd. The minimum detectable dose of Bovine Catalase was typically less than 1.95 IU/ml. The detection range was 7.8-500 IU/ml.

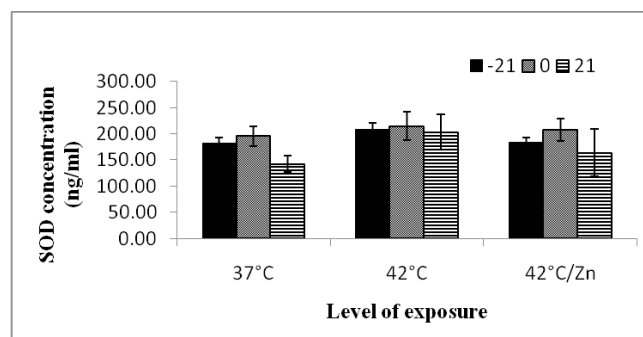


Figure 1. Changes in SOD (ng/ml) under thermal stress and Zinc treatment in Sahiwal transition cows

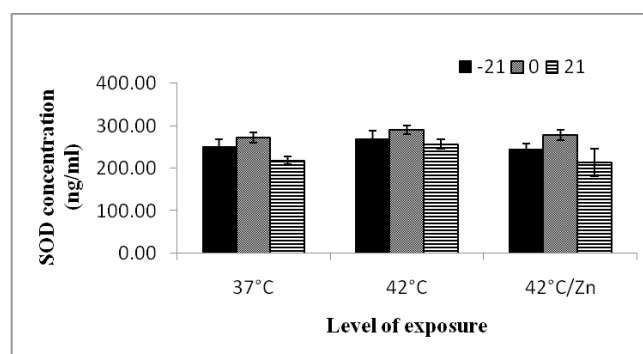


Figure 2. Changes in SOD concentration (ng/ml) under thermal stress and Zinc treatment in Karan Fries transition cows

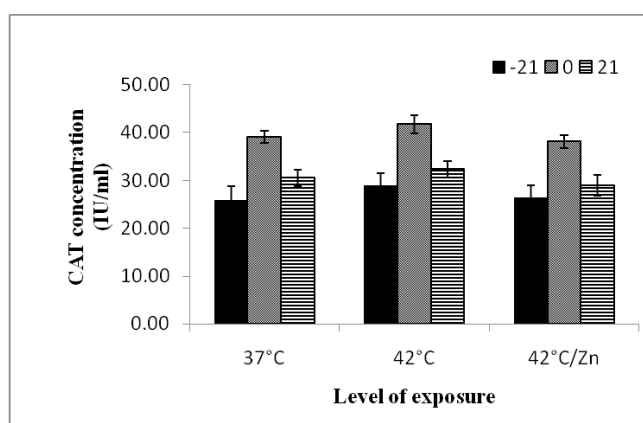


Figure 3. Changes in Catalase (IU/ml) under thermal stress and Zinc treatment in Sahiwal transition cows

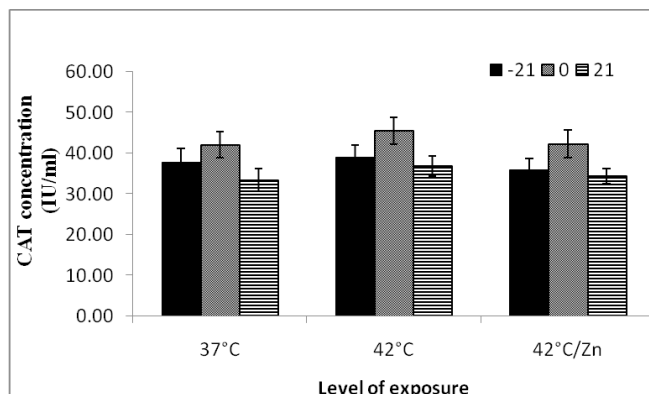


Figure 4. Changes in Catalase (IU/ml) under thermal stress and Zinc treatment in Karan Fries transition cows

Statistical Analysis

The analysis of the data was performed using the SAS software (SAS Enterprise Guide 9.3 version) programme by three factor analysis of variance for breed * days (peripartum) * *in vitro* treatment interaction using a correlation coefficient at 0.05 level of significance. Data was expressed as Least square mean (LSM).

$$Y_{ijk} = \mu + Expo_i + Br_j + Days_k + Expo * Breed + Br * Days + Days * Expo + Day * Expo * Breed + \epsilon_{ijk}$$

Y_{ijk} = kth observation on ith level of exposure with jth breed

μ = overall mean

Expo_i = effect of ith level of exposure

Br_j = effect of jth breed

Days_k = effect on kth day

ε_{ijkl} = random error

RESULTS AND DISCUSSION

The changes in the concentration of SOD have been presented in the Table 1 and are graphically represented in the figures 1 and 2. The SOD is significantly (P<0.05) higher in the PBMC of Karan Fries than the Sahiwal cows with concentration of 255.03±16.67 and 188.42±9.95 ng/ml, respectively. During the transition period, the concentration of SOD was highest on the day of calving in both the breeds at all the levels of exposure. Moreover, the concentration was highest at 42°C as compared to 37°C and 42°C/Zinc in both the breeds on all the days. The

concentration (291.19±10.30 ng/ml) of SOD was highest in KF on the day of calving exposed to 42°C whereas, the concentration (142.97±15.97 ng/ml) was least in the PBMC of Sahiwal at 37°C during the postpartum period. Zinc treatment decreased the concentration of SOD that otherwise increased while exposing the PBMC to 42°C throughout the transition period in both the breeds.

The values of SOD showed an increasing trend on the day of calving and during heat stress. These observations are in agreement with the observations of Maurya *et al.* (2014) who reported an increase in the SOD activity on the day of calving with the levels being lower in the peripartum state. The levels were also lower in the zinc treated group as they were in the Vitamin E supplemented group during the experiment conducted by Maurya *et al.* (2014). Contrary to this, Aggarwal *et al.* (2013) showed a decline in the SOD activity towards the day of parturition which further decreased in the postpartum phase. The α-tocopherol treatment increased the levels of SOD on the respective days. Bernabucci *et al.* (2005) also reported decreased SOD activity towards calving, and after calving, SOD activity rapidly declined. In the present study, the SOD activity increased probably to combat the effect of free radicals and to improve cell survival. The levels of SOD are more in KF as compared to Sahiwal which might be due to the fact that KF has some exotic inheritance thereby making it more susceptible to heat stress. More free radicals are formed in KF in response to heat and other calving related stress. To combat them, more SOD is produced. Kumar *et al.* (2011b) reported an increase in SOD level in caprine during thermal stress. Singh *et al.* (2014) found that high temperature did not increase ROS production significantly in Tharparkar but increased significantly (P<0.001) in Karan Fries cattle. The higher level of cytotoxicity seen in crossbred KF cows indicated their susceptibility to hot dry environment as compared to zebu cows.

The changes observed in the concentration of Catalase during the course of experiment are represented in the Table 2 and graphically depicted in the figures 3 and 4. Catalase showed a variation in relation to the type of exposure (37°C, 42°C and 42°C/Zn) given to the PBMC in culture. It also varied breed wise. The transition period also has the influence on the concentration of catalase. The concentration of Catalase was significantly (P<0.05) higher in the PBMC of KF than Sahiwal cows. The concentration in KF and Sahiwal was 38.60 ± 1.17 and



32.45 ± 1.71 IU/ml, respectively. The concentration of catalase was more on the day of calving at all the levels of exposure in both the breeds. Likewise, catalase showed more concentration when the PBMC were exposed to a temperature of 42°C on all the days in both the breeds. The concentration (25.90 ± 2.96 IU/ml) was minimum in Sahiwal on the day 21st prepartum and the exposure at 37°C. The highest concentration (45.59 ± 3.28 IU/ml) was found in KF at 42°C on the day of parturition. In all the cases, Zinc decreased the levels of catalase.

The Catalase showed the highest values on the day of calving and during the heat stress mainly to neutralize the excessive amount of H₂O₂ during the process. The Catalase concentration might increase because higher levels of SOD during stress cause more production of H₂O₂ to be neutralized by catalase. Maurya *et al.* (2014) also showed the same trend around parturition with the levels being highest on the day of calving. The concentration of catalase decreases while supplementing Vitamin E to the animals like it decreased when zinc was supplemented to the PBMC culture in the present study. The results in the present study are contrary to those shown by Aggarwal *et al.* (2013) in which the catalase concentration declined from prepartum to postpartum period in the transition Karan Fries cows. Further, it was shown that the concentration of Catalase increased while supplementing α -tocopherol in the respective groups. Bernabucci *et al.* (2005) and Sordillo *et al.* (2007) showed a relationship between the physiological changes associated with parturition and a loss in overall antioxidant potential was established in both humans and dairy cows. The possibility that oxidative stress during the transition period particularly during parturition may be a major underlying cause of inflammatory and immune dysfunction in dairy cattle has earlier been supported by various studies conducted either *in vivo* and *in vitro* (Sordillo and Aitken, 2009). A positive correlation (P<0.05) existed between SOD and Catalase.

CONCLUSION

The results of the current study indicated that zinc supplementation improves the antioxidant status and thus ameliorates the oxidative stress which otherwise supervenes during heat stress in transition period.

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