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# Mn-SOD and Cu,Zn-SOD mRNA expression in relation to physiological indices of Sahiwal and Karan-Fries Heifers under different temperature humidity indices*

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## ABSTRACT

The present study was carried out during October 2007 to July 2008 in five different combinations of maximum temperature (Tmax) / minimum temperature (Tmin) *viz.* P1: <20°C/<10°C; P2: >20°C/<10°C, P3: >30°C/<15°C; P4: >35°C/<20°C and P5: >35°C/>20°C. The study aimed to investigate the expression pattern of superoxide dismutase enzymes *viz.* Mn-SOD mRNA and Cu,Zn-SOD mRNA in relation to physiological responses of Sahiwal and Karan-Fries cattle. Physiological indices revealed no significant breed differences indicating similar levels of heat adaptability. During P1, expression of Mn-SOD was higher in KF than in Sahiwal while during P5, expression of Cu,Zn-SOD was higher in KF than in Sahiwal ($p<0.05$). Mn-SOD expression in Sahiwal increased with cold stress while Cu,Zn-SOD expression in KF increased with hot stress ($p<0.05$).

**Key words :** Mn-SOD, Cu/Zn-SOD mRNA, THI, Karnal, Sahiwal, Karan-Fries

Thermal stress occurs due to any single or a combination of environmental factors when the effective temperature of the environment is higher than the animal's thermoneutral zone. The heat stress impinging on the animal causes a chain of physiological, anatomical and behavioural changes leading to a reduction in productive functions. The reduction of productivity with devastating economic consequences to the global dairy industry due to warm environment has been documented (Bernabucci *et al.,* 2010).

Thermal stress can also lead to oxidative stress in a living organism as a result of imbalance between the production of reactive oxygen metabolites and the capacity of the antioxidant mechanism to neutralize these reactive oxygen species (Sies, 1997). Heat stress induced increase in SOD enzyme activity had been well documented (Bernabucci, *et al.,* 2002; Yatto *et al.,* 2014). There was increase in expression of Mn-SOD and Cu,Zn-SOD mRNA in response to oxidative stress caused by immobilization (Oishi and Machida, 2002) and expression of Cu,Zn-SOD mRNA had been found to correlate the degree of thermotolerance of bovine embryos (Lazzari *et al.,* 2002). It had been well documented that Zebu cattle breeds adapted to hot climatic conditions were less affected by thermal stress as compared to cattle of European origin (Hansen, 2004; Lacetera *et al.,* 2006).

In the present study attempts had been made in order to study the *in-vivo* expression of Mn-SOD mRNA and Cu,Zn-SOD mRNA in Sahiwal and crossbred cattle Karan-Fries (KF) in relation to their normal physiological indices during different temperature conditions. The study will add in better understanding of the cellular responses involved in the better thermotolerance of Zebu cattle compared to other cattle breeds of exotic origin as well as in estimating the impact of global climate change on the performance of the livestock animals.

## MATERIALS AND METHODS

Sahiwal and KF heifers numbering 6 each were selected from the herd maintained at National Dairy Research Institute (NDRI), Karnal, India. The animals were in the age group of 2-2.5 years and average body weight was 301.3±6.91kg. The animals were given a maintenance concentrate mixture @1kg per animal in addition to *ad lib* roughages and water as per Kearl's standard (Kearl, 1982). Concentrate mixture consisted of mustard cake, maize, wheat bran, rice bran, mineral mixture and salt. The CP and TDN in diet was 12% and 60% respectively. The study was carried out in five different combinations of Tmax and Tmin which were selected based on the climatograph prepared from the records of previous 10 years (Table 1).

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**Table 1:** Different cominations of maximum (Tmax) and minimum (Tmin) temperature considered under study.

| Period No. | Temperature (°C) Tmax | Tmin | Duration |
|---|---|---|---|
| P1 | < 20 | <10 | 27th December, 2007 to 2nd January 2008 |
| P2 | >20 | <10 | 7th December to 13th December, 2007 |
| P3 | >30 | <15 | 25th October to 31st October, 2007 |
| P4 | >35 | <20 | 11th to 21st April, 2008 |
| P5 | >35 | >20 | 29th June to 6th July, 2008 |

Prevailing temperature in Karnal as recorded at Central Soil Salinity Research Institute (CSSRI), Karnal were obtained. Everyday records of minimum, maximum ambient temperature, dry bulb and wet bulb temperature recorded at I: 0722\ 0830 and II: 1422 h IST. The Temperature humidity index (THI) was calculated as per the formula given by National Research Council (1971).

$$THI = 0.72(Tdb + Twb) + 40.6$$

Where, Tdb and Twb are dry bulb and wet bulb temperatures respectively.

The physiological indices *viz*, respiration rate (RR), rectal temperature (RT) and heart rate (HR) were recorded in between 0600 h IST and 0700 IST during the whole period of study. RR (breaths $min^{-1}$) was recorded by observing the flank movements. RT (°C) was recorded by using a digital thermometer. HR was calculated from the electrocardiograms of individual animals by using bipolar limb leads of Student Physiograph. The vertical sensitivity was adjusted to give 10 mm deflections per mV and the paper speed was kept at 25 mm $s^{-1}$. In order to find out heat adaptability co-efficient, Dairy search index (DSI) was calculated from the formula given by Thomas *et al.* (1973).

$$DSI = 0.5\frac{x1}{x} + 0.2\frac{y1}{y} + 0.3\frac{z1}{z}$$

Where x1, y1 and z1 were the observed RT, RR and HR, respectively while x, y and z were normal/expected RT, RR and HR rate, *i.e.* 38.3°C, 23 breaths $min^{-1}$ and 78 beats $min^{-1}$, respectively.

Two ml each of blood samples were collected for separation of peripheral blood mononuclear cells (PBMC) and subsequent isolation of total RNA. PBMC were separated by gradient centrifugation with Histopaque (Sigma). The PBMC were cultured by incubation at 38°C for 3h in lymphocyte selective media, RPMI-1640 supplemented with penicillin @ 100 mg $ml^{-1}$, streptomycin @ 100 mg $ml^{-1}$ and bovine serum albumin @ 0.5% to obtain the lymphocytes. The lymphocytes were collected by centrifugation and dried to make pellets. The cell pellets were stored at -20°C until RNA isolation. Total RNA was isolated by using Tri reagent (Sigma) as per the guidelines with slight modifications. After determining the purity and quality of RNA, the RNA samples were reversed transcribed to cDNA by using RevertAid First Strand cDNA synthesis kit (Fermentas). From the reversed transcribed cDNA PCR was carried out for Mn-SOD and Cu,Zn-SOD genes by using specific primers. The GAPDH gene was used as a house keeping gene for relative measure of expression of desired genes. The details of the primers used in the experiment are presented in Table 2.

The PCR of Mn-SOD cDNA and Cu,Zn-SOD cDNA and subsequent record of amplified products by SDS-PAGE were carried out by following the methods as described by Sambrook *et. al.* (1989) with slight modifications. The PCR reaction mixture (25µl) consisted of 2.5 µl 10x Tag polymerase buffer, 4µl 25 mM $MgCl_2$, 0.5 µl 10 mM dNTP, 1µl 10 mM forward primer, 1µl 10 mM reverse primer, 2µl cDNA, 0.15 µl 5 $U^{-1}$ µl Taq polymerase and 13.85µl nuclease free water. The amplified products of PCR were electrphoresed in agarose gel containing ethidium bromide (5µg 100$ml^{-1}$ gel). Semi quantitative measure of expression of mRNA of genes of interest was obtained from the digital pictures recorded from the electrophoresed products. The integrated density value (IDV) of each of the bands was measured in GelDoc (Image AIDE 10990*Syn1234* mpcs5870337c Spectronics, Gel Doc Software). Expression of mRNA of gene of interest was obtained from the ratio of IDV of specific gene to IDV of GAPDH.

The data were analyzed by using SYSTAT VERSION 6.0.1, COPYRIGHT (C) 1996, SPSS Inc., Chicago, IL. The one way analysis of variance was carried to find out the effect of periods and breed. Fisher's Least-Significant-Difference Test was applied to find out matrix of pair wise comparison probabilities between different groups.

## RESULTS AND DISCUSSION

The variation in prevailing Tmax/Tmin, Tdb, Twb and the measure of THI recorded during the period of study are

**Table 2:** Details of Mn-SOD, Cu,Zn-SOD and GAPDH genes

| Sl. No. | Gene | Primer sequence | $T^A$ (°C) | Amplicon size (bp) | Gene bank accession number | Reference |
|---|---|---|---|---|---|---|
| 1 | Mn-SOD | 5'- CCCATGAAGCCTTTCTAATCCTG-3'<br>5'-TTCAGAGGCGCTACTATTTCCTTC-3' | 64 | 307 | L22092.1 | Lonergan *et al.*, (2003) |
| 2 | Cu,Zn-SOD | 5'-AAGGCCGTGTGCGTGCTGAA-3'<br>5'-CAGGTCTCCAACATGCCTCT-3' | 60 | 246 | Y00404 | Lazzari *et al.*, (2002) |
| 3 | GAPDH | 5'-CCCATCACCATCTTCCAGG-3'<br>5'-AGTGAGCTTCCCGTTCAGC-3' | 54 | 471 | - | Correa *et al.*, (2007) |

**Table 3:** Ambient temperature and THI during different periods

| Parameters | | Periods | | | | |
|---|---|---|---|---|---|---|
| | | P1 | P2 | P3 | P4 | P5 |
| T(°C) | Max | 16.7 | 20.4 | 30.8 | 36.0 | 37.7 |
| | Min | 2.5 | 9.0 | 13.6 | 17.8 | 23.1 |
| | Average | 9.6 | 14.7 | 22.2 | 26.9 | 30.4 |
| $T_{db}$ (°C) | Max | 18.4 | 19.8 | 30.5 | 36.2 | 35.7 |
| | Min | 3.6 | 10.4 | 14.9 | 20.5 | 27.0 |
| | Average | 11.0 | 15.1 | 22.7 | 28.3 | 31.3 |
| $T_{wb}$(°C) | Max | 11.4 | 14.8 | 20.1 | 20.7 | 24.4 |
| | Min | 2.8 | 9.5 | 14.3 | 17.4 | 22.9 |
| | Average | 7.1 | 12.1 | 17.2 | 19.0 | 23.7 |
| THI | Max | 62.0 | 65.5 | 77.0 | 81.5 | 83.9 |
| | Min | 45.2 | 54.8 | 61.6 | 67.8 | 76.5 |
| | Average | 53.6 | 60.2 | 69.3 | 74.7 | 80.3 |

**Table 4:** Effect of different periods on RR (breaths $min^{-1}$), RT (°C), HR (beats $min^{-1}$) and DSI of Sahiwal and Karan-Fries cattle

| Parameter | Breed | Periods | | | | |
|---|---|---|---|---|---|---|
| | | P1 | P2 | P3 | P4 | P5 |
| RR | Sahiwal | $31\pm1^{b}$ | $27\pm1^{ab}$ | $25\pm2^{a}$ | $33\pm2^{b}$ | $54\pm3^{c}$ |
| | KF | $31\pm1^{a}$ | $36\pm2^{a}$ | $31\pm3^{a}_{*}$ | $34\pm1^{a}$ | $57\pm3^{b}$ |
| RT | Sahiwal | $38.64\pm0.09^{a}$ | $38.77\pm0.03^{a}$ | $38.76\pm0.11^{ab}$ | $38.95\pm0.14^{b}$ | $39.33\pm0.08^{c}$ |
| | KF | $38.64\pm0.11^{a}$ | $38.69\pm0.08^{a}$ | $38.77\pm0.12^{a}$ | $38.79\pm0.07^{a}$ | $39.25\pm0.08^{b}$ |
| HR | Sahiwal | $80\pm5^{ab}$ | $77\pm5^{a}$ | $89\pm9^{abc}$ | $95\pm5^{bc}$ | $96\pm4^{c}$ |
| | KF | $88\pm4^{b}$ | $82\pm3^{ab}$ | $73\pm7^{a}$ | $81\pm8^{ab}$ | $96\pm2^{c}$ |
| DSI | Sahiwal | $1.08\pm0.02^{b}$ | $1.03\pm0.02^{a}$ | $1.06\pm0.04^{a}$ | $1.16\pm0.03^{b}$ | $1.35\pm0.03^{c}$ |
| | KF | $1.11\pm0.02^{a}$ | $1.13\pm0.03^{a}$ | $1.05\pm0.05^{a}$ | $1.11\pm0.03^{a}$ | $1.38\pm0.02^{b}$ |

Values are mean ± SE; means of same breed with different superscripts differ significantly from each other ($p<0.05$); * indicates significant difference between the breeds ($p<0.05$)

presented in Table 3. The Tmax/Tmin recorded in different periods were found to fall in the ranges as per the climatograph of the previous 10 years.

In intensive animal housing with environmental modification, temperature alone is also the usual control parameter. However, by combining temperature and humidity effects, the temperature humidity index (THI) does capture much of the impact of warm to hot thermal environments on animals (Hahn *et al.*, 2009). The THI is commonly used as an indicator of the intensity of climatic stress on animals,

where a THI of 72 and below is considered as no heat stress, 73–77 as mild heat stress, 78–89 as moderate, and above 90 as severe (Thom, 1959). On the other hand, the Livestock Weather Safety Index (LCI, 1970) categories associated with THI are normal (THI ≤ 74), alert (75–78 THI), danger (79–83 THI) and emergency (THI ≥ 84). As per the record of THI in different Tmax/Tmin conditions (Table 3), the P1, P2 and P3 were not stressful to the animals and P4 and P5 were stressful for the animals.

RR during heat exposure is known to increase more rapidly than other responses and often occurs at a lesser critical ambient temperature than other responses such as RT or changes in feed intake (Hahn, 1999). In addition, RR (*i.e.,* an indicator of respiratory evaporative heat loss) is one of several effecter responses, including sweat rate and peripheral vasodilation that determine the internal body temperature in response to heat stress. In theory, it is only when the avenues for heat loss are compromised, or limits of effectiveness are reached, that there would be an increase in internal body temperature (Scharf *et al.,* 2010). The effect of heat stress on RR was recorded in both the breeds (Table 4). Sahiwal cattle recorded higher RR in P1 compared to P2 and P3 which might be due to cold stress. It could be predicted that during the peak winter when Tmin recorded was 2.5°C, Sahiwal heifers suffered cold stress while KF heifers were not affected. The record of higher DSI of Sahiwal in cold stress might be due to the increase in RR of Sahiwal during cold stress unlike the KF in which RR did not change with cold stress. When the cold receptors are activated heat production mechanism are to be stimulated which includes increase in feed intake in order to increase the basal metabolic rate (BMR). As Sahiwal being a native breed, its increase in RR might be a compensatory response to increase in BMR during cold exposure. KF heifers were however found to have stable RR except in extreme hot condition during P5 ($p<0.05$) when average THI was 80.3. Such differences could be due to variations in their genetic make-up as evident from the record of higher RR in KF during P3 ($p<0.05$) when the Tmax/Tmin was optimal and THI was 69.3.

Another most commonly used variable to assess heat tolerance is RT. Because RT is easy to measure, well documented and it makes a reliable index. Above a threshold environmental condition, RT begins to increase as a result of the animal's inability to adequately dissipate the excess heat load by increased respiratory vaporization (Scharf *et al.,* 2010). In the present study, an average THI of 80.3 recorded during P5 caused rise in RT ($p<0.05$) in both the cattle breeds (Table 3, 4). The rise in RT was however not influenced by breed indicating that both the cattle breeds responded similarly in increasing RT.

HR is another instantaneous measure of sympathetic and parasympathetic activity in animal body. Record of HR had been indicated to be a non invasive means for detection of presence of stress in cattle (Lefcourt *et al.,* 1999). Normal heart rate of Sahiwal and KF varied in a wide range with no significant effects of the breed. Sahiwal and KF heifers almost exhibited siilar patterns in increasing the HR during heat stress. It was evident that average THI of 80.3 recorded during P5 increased HR in both Sahiwal and KF ($p<0.05$) as compared to conditions when average THI was 60.3 in Sahiwal and 69.3 in KF which were indicators for heat stress (Lefcourt *et al.,* 1999; Berman, 2005). The measurement of heart rate variability (HRV) had been indicated to be a non-invasive approach to measure stress in calves and cows (Mohr, *et al.,* 2002; von Borell *et al.,* 2007). The HRV as calculated from the ECG records of the animals in the present study (data not presented here) however revealed no significant differences between the breeds and the different THIs indicating that the changes in the heart rates were within the normal physiological ranges in both the breeds.

The two breeds showed no significant difference in RR, RT and HR in different temperature conditions unlike reports of Gaughan *et al.* (1999) when crossbred cattle had higher RR and RT than Zebu cattle when exposed to extreme heat stress. An ability to increase RR and sweating are characteristics for Zebus to dissipate heat through evaporative cooling. The records of similar response of Sahiwal and KF in terms of their RR, RT and HR when the animals were under heat stress conditions pointed to the assumptions that KF had also become heat tolerant like Sahiwal. Further heat tolerance index as measured by DSI indicated that Sahiwal heifers had higher DSI during P1 and P4 as compared to that during P2 and P3 and highest DSI during P5 while KF heifers had higher DSI in P5 than other temperature conditions.

In order to detect presence of cold or heat stress in Sahiwal and KF, further investigation was carried out to identify a stress marker. The effect of different temperature conditions on expression of Mn-SOD mRNA were evident ($p<0.05$) in both the breeds (Fig. 1). A mild degree of stress experienced during extreme cold was evident in both the breeds with higher Mn-SOD expression during P1 when

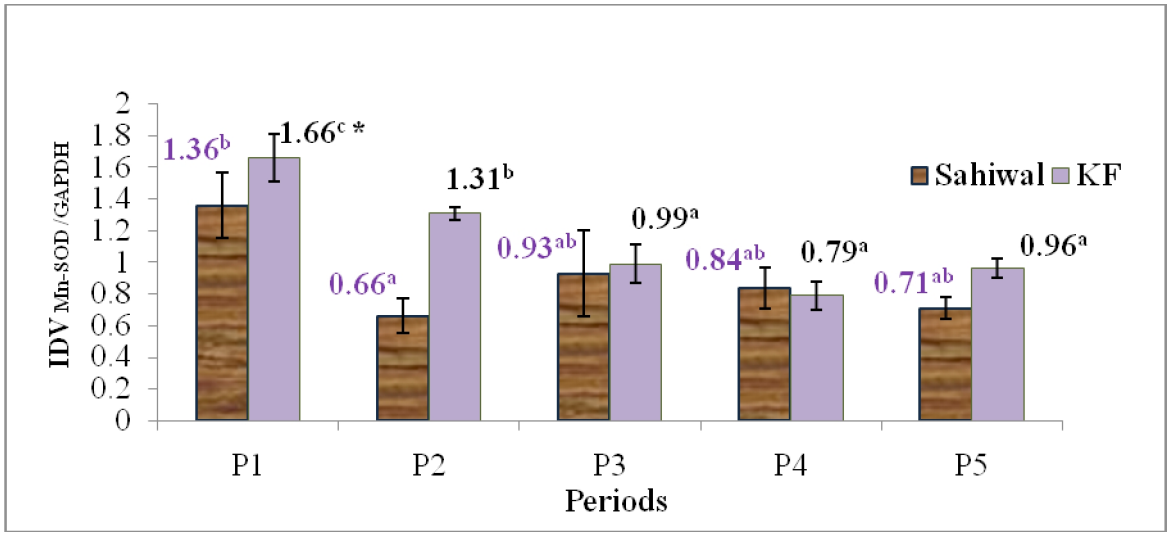


**Fig. 1:** Mn-SOD mRNA expression in Sahiwal and KF during different periods

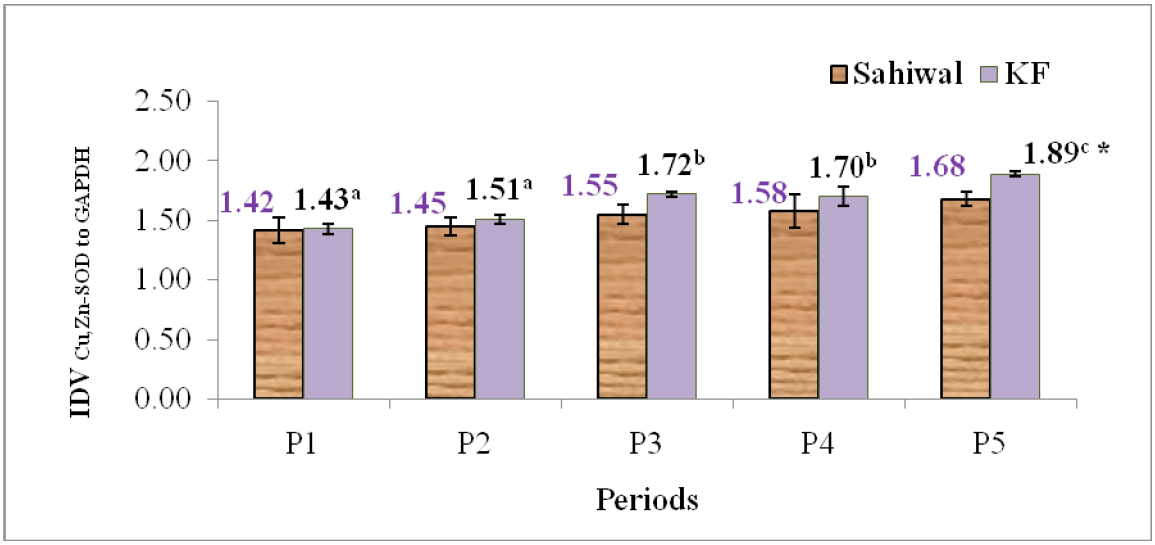


**Fig. 2 :** Cu,Zn-SOD mRNA expression in Sahiwal and KF during different periods

Values are mean ± SE; means of same breed with different superscripts differ significantly from each other ($p<0.05$); * indicates significant difference between the breeds ($p<0.05$)

average Tmin was 2.5°C as compared to rest of the other periods ($p<0.05$). The expression of Mn-SOD during P1 and P5 were higher in KF than in Sahiwal ($P<0.05$). SOD activity had been found to increase during thermal stress causing oxidative stress in numbers of studies (Bernabucci *et al.,* 2002). Apart from involvement of SOD in mechanisms against oxidative stress (Ellah *et al.,* 2009; Zlatkoviæ and Filipoviæ, 2011) expression of SOD had been detected to associate degree of thermotolerance in vitro (Loven *et. al.,* 1985). The ability to express more Mn-SOD in KF might be an indication for the adaptability of KF without significant changes in terms of their physiological responses during the periods of heat stress.

The expression of Cu,Zn-SOD mRNA in Sahiwal was not influenced by different THI (Fig. 2). In case of KF however, Cu,Zn-SOD expression increased with increase in THI ($p<0.05$) from P1 and P2 to P3 and P4 and further to P5 (Fig. 2). During extreme heat stress in P5 when THI was 80.26, Cu,Zn-SOD expression was found to increase in KF ($p<0.05$). Heat stress during P5 caused higher Cu,Zn-SOD expression in KF than in Sahiwal ($p<0.05$). Activity of CuZn-SOD had been known to increase in presence of oxidative stress (Ellah *et al.,* 2009) and the expression of CuZn-SOD varied in acute or chronic oxidative stress (Zlatkoviæ and Filipoviæ, 2011). CuZn-SOD activity had been found to correlate with thermotolerance in Chinese hamster ovary cells and carcinoma cell (Loven *et al.,* 1985). It could be possible that KF heifers showed physiological responses

similar to that of Sahiwal indicating similar thermotolerances. Cellular changes in ability to increase expression of Cu,Zn-SOD in KF during thermal stress higher than that in Sahiwal might be one genetic mechanism adopted by KF.

Thermal stress causes a number of cellular changes comprising of increase in production of heat shock proteins (HSPs) in order to protect the cells from other hazardous effects of oxidative stress. The HSPs had been found to have protective effects against oxidative stress. In our previous reports, Sahiwal had been found to have higher ranges of HSP72 than KF (Prava and Upadhyay, 2014; Prava *et al.,* 2015). Seasonal variation in HSP72 was recorded in KF, which increased significantly during heat stress in KF but not in Sahiwal (Prava *et al.,* 2015). The rat muscles with higher expression of HSP72 had been found to downregulate both Mn-SOD and Cu,Zn-SOD mRNA in response to oxidative stress (Selsby and Dodd, 2005). In our present finding, record of lower Mn-SOD and Cu,Zn-SOD in Sahiwal could be due to less activation of antioxidant mechanism in the presence of higher basal content of HSP70 in Sahiwal than in KF (Prava *et al.,* 2015).

From the present investigation it could be drawn that Sahiwal and KF exhibited significant differences in expression of Mn-SOD mRNA during cold stress and Cu,Zn-SOD mRNA during heat stress. It could also be presumed that Mn-SOD expression was more responsive to cold while Cu,Zn-SOD was more responsive to heat stress. The ability to activate the antioxidant mechanism in KF played an important role in maintaining physiological responses in both Sahiwal and KF within the normal ranges during different THIs.

## CONCLUSION

Thermal stress includes both heat stress, during extreme summer season as well as cold stress, during extreme winter season. Temperature determines metabolic rates, heart rates and other important factors within the bodies of animals, so an extreme temperature change can easily distress the animal body. The effect of high temperature is further aggravated when heat stress is accompanied by high ambient humidity. The physiological responses of Zebu cattle, Sahiwal and a crossbred of Sahiwal, KF were almost similar during different THIs showing similar levels of heat tolerances. Zebu cattle experienced a mild degree of cold stress in extreme cold temperature condition prevailed at Karnal, India. Genetic differences were exhibited in expression of two forms of SOD enzymes, Mn-SOD mRNA during cold stress and Cu,Zn-SOD mRNA during heat stress. Apart from the implications of SOD in determining the presence oxidative stress in the body systems, SOD enzyme expressions seem to play important roles in protection of cells against thermal stress.

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