



Heat stress amelioration by astaxanthin supplementation and Nrf2 transcribed downregulation of HSP70 and MnSOD in Karan Fries (Holstein Friesian ×Tharparkar) heifers

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ABSTRACT

Karan Fries (Holstein Friesian ×Tharparkar) heifers are sensitive to heat stress due to exotic inheritance. Therefore their productive performances are affected adversely under tropical climatic conditions. Hence, the present study was conducted on Karan Fries (10–12 months) heifers by dividing into two group i.e. control (fed as per ICAR, 2013) and treatment group (additionally supplemented with astaxanthin @ 0.25 mg/kgBW/day/animal). Environmental parameters (dry and wet bulb temperature, and relative humidity) were recorded throughout the study and temperature humidity index (THI) was calculated. THI was used to assess the levels of summer stress. Blood samples were collected at fortnightly interval from both the groups of heifers for estimation of oxidative stress markers i.e. antioxidants enzyme (SOD, GPx and catalase). RNA was isolated from collected blood samples for Nrf2, HSP70 and MnSOD gene expression at monthly interval. Plasma catalase, SOD and GPx levels were lower in astaxanthin supplemented group of Karan Fries heifers than control group. The expression of HSP70 and MnSOD genes was down-regulated and Nrf2 was up-regulated in treatment compared to control group. The THI had a positive correlation with oxidative stress markers i.e. antioxidants enzyme (SOD, GPx & Catalase) as well as expression pattern of HSP70, and MnSOD genes. Based on the results of the present study, it can be concluded that astaxanthin (potent antioxidant) supplementation was effective in lowering the stress levels in treatment group by down-regulating the genes related to heat stress. Therefore, astaxanthin can be an effective supplement for ameliorating the adverse effect of heat stress, mainly in heat sensitive livestock species.

Key words: Antioxidant enzymes, Astaxanthin, Gene expression, Karan Fries, Summer stress

Global warming coupled with the subtropical harsh environment in Indian subcontinent exposes the livestock species to thermal stress which affects the productive, reproductive and metabolic functions (Wankar *et al.* 2014). Heat stress occurs when the core body temperature of a given species exceeds the temperature range of the species specified for its normal activity (Ganaie *et al.* 2013). Heat stress leads to several metabolic dysfunctions that may result in a decline of animal performance. Maibam *et al.* (2017) reported that metabolic dysfunctions during heat stress leads to reactive oxygen species (ROS) induced cellular damage.

High environmental temperature challenges the homeostatic state of the animal and stimulates excessive production of free radicals. In response, the body tissue synthesizes and releases antioxidant enzymes and heat shock proteins (HSP) to protect itself from the deleterious cellular effects of ROS. The heat shock response is a coordinated genetic response to a wide range of environmental stressors culminating in the induction of genes encoding molecular chaperones, proteases, and other

proteins essential for maintaining cellular homeostasis to offset the damage associated with the stress. HSP70 is one of the major chaperones of the cell which plays a crucial role in guiding conformational status of the proteins during folding and translocation (Arya *et al.* 2007). Activation of p38MAPK by heat stress results in increased expression of MnSOD (Shin *et al.* 2008). Heat stress has been shown to activate p38MAPK upstream of HSP70 augmentation. The unfavourable effects of ROS are counteracted by the antioxidant defense of the cells which includes various antioxidant enzymes and ROS scavenging molecules. Of the various antioxidant enzymes, Cu/ZnSOD and MnSOD are primarily responsible for the conversion of O₂⁻ to H₂O₂, which is then degraded by catalase or peroxidases. The cytoprotective genes HSP70 and MnSOD have been found to be over expressed during oxidative stress to counteract the elevated ROS levels.

Studies with antioxidant supplementation demonstrate inhibition in free radical generation in response to oxidative stress in animals. Astaxanthin being potent herbal phytochemical antioxidant helps in ameliorating the heat stress by scavenging the free radicals. Antioxidant activity of astaxanthin is 40 times higher than β-carotene and lutein

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and 100 times higher than α -tocopherol against reactive oxygen species (Kristinson and Miyashita 2014). The mechanisms underlying the expression of survival genes by heat stress remains largely undiscovered. Therefore, the present study was undertaken to identify the signalling elements upstream of the upregulated survival genes in heat stress.

MATERIALS AND METHODS

Ethical approval: The experiment was approved by the Institutional Animal Ethics Committee constituted as per the Article No. 13 of the CPCSEA rules, laid down by Government of India.

Study area: The experiment was conducted in the cattle yard of the Institute. Karnal is situated at an altitude of 250 m above mean sea level and at 29°42'N latitude and 79°59' E longitudes. The highest temperature goes up to 45°C during summer and minimum temperature 3.5–4°C during winter. The average rainfall is about 700 mm.

Selection of animals and their management: The study was conducted on 12 Karan Fries heifers (Holstein Friesian $\times$ Tharparkar) of 10–12 months. These animals were further divided into two groups equally i.e. control and treatment. All the animals were maintained under standard managerial conditions followed at ICAR-NDRI livestock farm. The nutrient requirement of all the animals was met by feeding *ad lib.* green fodder and 1 kg concentrate. Green fodder predominately consisted of maize, jowar, oat and was fed after chaffing. The ingredients of concentrate mixture were prepared to provide 20% crude protein and 70% total digestible nutrients. Prior to experimental period, mass deworming of animals was done and during experimental period routine health management of the animals were also followed.

Blood collection and analysis: Blood samples were collected from all the experimental animals at fortnightly intervals in heparinised vacutainer tube. Plasma was separated through centrifugation of blood tubes @ 3000 rpm for 25 min. The blood plasma was stored in small aliquots in eppendorff tubes at -20°C for further analysis. Lymphocytes were separated from the buffy coat to study the expression of the genes related to transcription factor (Nrf2), heat stress (HSP70) and antioxidant (MnSOD) at monthly interval.

ELISA for antioxidant enzymes: Superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) were estimated in blood plasma samples using—Bovine SOD ELISA Kit; Bovine GSH-Px ELISA Kit; and Bovine CAT ELISA Kit, respectively supplied by Bioassay Technology, China.

Total RNA isolation and cDNA synthesis: Total RNA was prepared using RNeasy Mini Kit (Qiagen India Pvt. Ltd.) according to manufacturer's protocol. RNA was dissolved in nuclease free water and the purity of RNA was verified by optical density (OD) absorption ratio OD 260/280 nm using Biospec spectrophotometer (Shimadzu Corp., Kyoto, Japan). RNA integrity was assessed in 1.5% agarose gel

electrophoresis by observing rRNA bands corresponding to 28S and 18S. For each sample, 200 ng of total RNA was used for cDNA synthesis using Revert Aid First strand cDNA synthesis kit (Thermo Scientific, Rockford, IL, USA) by reverse transcription PCR according to the manufacturer's protocol. The prepared cDNA was analyzed using PCR and stored at -80°C until further use for quantitative real time PCR. The primers were designed using primer 3 software from NCBI database.

Quantitative real-time PCR (qRT-PCR): Polymerized chain reaction was performed to amplify the target and reference genes on Real Time Thermocycler 7500 (Applied Biosystem) with FastStart DNA Master SYBR Green I mix (Bio-Rad, Hercules, CA, USA) by following manufacturer's instructions. The sequence of primers, annealing temperatures, and size of gene fragments amplified are shown in Table 5. Along with the target genes, two housekeeping genes [glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β -actin] were amplified for relative expression measurements. However, the expression data of GAPDH was taken as an endogenous reference. Each sample was run in duplicates, and in all the cases, nuclease free water was substituted for template in negative controls. Relative quantification of a target gene was done by comparing the expression levels of reference gene (GAPDH), as per the method of Livak and Schmittgen (2001).

Statistical analysis: Data were analyzed by analysis of variance using SAS software, version (9.1). Results were expressed as the means $\pm$ SEM. A difference with value $P \leq 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The THI was calculated using dry and wet bulb temperature at fortnightly interval during experimental period to assess the adverse effect of heat stress on Karan Fries heifers (Table 1). As the levels of THI increased, the oxidative biomarker also increased (Table 4).

Oxidative stress markers: SOD was significantly ($P \leq 0.05$) higher in control group compared to treatment group. GPx in control group was significantly ($P \leq 0.05$) higher (67.10 ± 1.00 U/ml) than treatment (57.60 ± 0.68 U/ml) group. The catalase activity in control and treatment group was 19.00 ± 0.34 ng/ml and 17.55 ± 0.22 ng/ml, respectively. The SOD, GPx and catalase activity increased with the increase in ambient temperature and THI (Table 4).

Superoxide dismutase is an important endogenous antioxidant enzyme that scavenges ROS (Tan *et al.* 2012). The results of the present study showed significant higher activity of superoxide dismutase in control group than the treatment group. The major defense in detoxification of superoxide and hydrogen peroxide are SOD, CAT and GPX. SOD breakdown superoxide (O_2^-) to H_2O_2 and dioxygen. The results of the present study are in agreement with Soren *et al.* (2017) who reported similar trend in SOD levels in seminal plasma of Karan Fries bulls i.e. significantly ($P \leq 0.05$) lower levels of SOD in astaxanthin (AX) treated

Table 1. Mean fortnightly temperature humidity index (THI) during the experimental period

Month	Fortnights	THI _{avg}
April	I	73.82±0.73
	II	78.48±0.57
May	III	79.71±0.58
	IV	80.19±0.55
June	V	81.51±0.74
	VI	79.94±0.73
July	VII	82.03±0.44
	VIII	81.77±0.36
August	IX	81.33±0.32
	X	81.13±0.35
September	XI	80.08±0.34
	XII	78.48±0.44

Table 2. Influence of astaxanthin supplementation on SOD, GPx and catalase of Karan Fries heifers during summer

Parameter	Control	Treatment
SOD (ng/ml)	22.85 ^X ±0.27	18.00 ^Y ±0.20
GPx (U/ml)	67.10 ^X ±1.00	57.60 ^Y ±0.68
Catalase (ng/ml)	19.00 ^X ±0.34	17.55 ^Y ±0.22

The values are mean±SE of 12 observations on six animals. The values with different superscripts within the rows differed significantly ($P\leq 0.05$).

group than control groups during hot dry, hot humid, spring and winter season. Lallawmkimi *et al.* (2010) studied the effect of seasons on antioxidant status of heifers and reported significantly higher SOD levels during summer compared to winter. In our study, the lower levels of SOD in treatment group of animals might be due to antioxidant capacity of AX, which neutralizes the effect of higher ROS production and causes breakdown of superoxide (O_2^-) to H_2O_2 and oxygen during heat stress. Yin *et al.* (2018) reported that SOD activity was significantly reduced ($P\leq 0.05$) to 8.6% for vitamin C and 22.3% lower for sodium bicarbonate (Vitamin C-Na) ($P\leq 0.01$) supplementation to relieve heat stress in H9C2 cells. The positive correlation of SOD with THI in our study are in accordance with Chaudhary *et al.* (2015) who also revealed the positive association of SOD with THI in Surti buffaloes.

The mean values of glutathione peroxidase in control group was significantly ($P\leq 0.05$) higher compared to treatment group during the summer stress. Lallawmkimi *et al.* (2010) also reported significantly higher GPx levels during summer than winter in buffaloes. Kumar *et al.* (2015) reported concentration of GPx as 2.71 and 2.34 nmol/min/ml in control and chromium propionate (antioxidant) supplemented group of Sahiwal cows, respectively. In our study, the lower levels of GPx in supplemented group might be due to antioxidant capacity of AX, which neutralizes the effect of ROS produced during heat stress. GPx protects cells against devastating oxidative stress. In the present study, similar pattern of GPx was observed as that of SOD, because action of SOD on superoxide ions resulted into

increased production of $H_2O_2^-$, which is neutralized by coordinated increase in GPx. GPx activity is connected to enhance the tolerance capacity of animals to thermal stress and prevents from protein denaturation (Singh *et al.* 2014).

Catalase is a tetrameric iron porphyrin protein located in the peroxisome and acts on H_2O_2 and catalyze into H_2O and O_2 . Chandra and Aggarwal (2009) reported higher catalase activity in prepartum crossbred cows during summer (159.94 ± 0.10 $\mu\text{mol/min/mgHb}$) than winter (153.85 ± 0.08 $\mu\text{mol/min/mgHb}$). Catalase levels were significantly ($P\leq 0.05$) higher in control group than the treatment group. The results of present study are in general agreement with that of Sheikh *et al.* (2015) who reported complementary relationship of catalase with the SOD, since SOD acts on free radicals and releases H_2O_2 and after that catalase neutralizes H_2O_2 into non-toxic product. Antioxidant like melatonin and might be astaxanthin also exerts its protective action against thermal stress by scavenging oxidative and nitrosative free radicals (Reiter *et al.* 2009). Shibu *et al.* (2008) reported higher ($P\leq 0.05$) catalase activity during exposure to heat stress in Karan Fries cattle. Contrary to our results, Singh *et al.* (2016) reported increasing trend of antioxidant enzymes like GPx, GR, SOD and catalase in summer anoestrus buffaloes treated with exogenous melatonin. The probable reason for the contradictory results might be due to the levels of stress, age of the animals and species.

Relative mRNA expression of Nrf2, HSP70 and MnSOD gene: The values of Nrf2 expression in treatment group (3.55 ± 0.218) was significantly ($P\leq 0.01$) higher than control (1.92 ± 0.086) group. Likewise, mRNA expression of HSP70 showed significantly ($P\leq 0.05$) higher levels in control group compared to treatment group. The expression of MnSOD gene in control (5.08 ± 0.274) was significantly ($P\leq 0.05$) higher than treatment (2.99 ± 0.197) group.

Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a key transcription factor which controls the cellular antioxidant response against oxidants (Kobayashi *et al.* 2006). In our study, the mean mRNA expression of Nrf2 was significantly ($P\leq 0.01$) higher in treatment than control group. The higher expression in treatment group might be due to involvement of astaxanthin in Nrf2-ARE pathway inducing Nrf2 nuclear localization by activation of the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway, which causes induction of Phase II enzymes (NQO1, HO-1, GCLM, and GCLC) and protect the cell from oxidative stress (Li *et al.* 2013). Astaxanthin is a powerful free radical scavenger, which protects the cells from oxidative damage (Lee *et al.* 2011). Dietary phytochemicals like EGCG (Sahin *et al.* 2010) and curcumin (Shen *et al.* 2006) activated Nrf2 and induced expression of antioxidant or phase two detoxifying enzymes. The results of present study are in agreement with Sahin *et al.* (2010), Sahin *et al.* (2011) and Sahin *et al.* (2012) who reported higher Nrf2 expression (59%) by supplementation of phytochemical antioxidant (EGCG, lycopene and resveratrol) and these Nrf2 expressions was

Table 3. Influence of astaxanthin supplementation on expression pattern of HSP70 and MnSOD genes of Karan Fries heifers during summer

Parameter	Control	Treatment
Nrf2	1.92 ^X ±0.086	3.55 ^Y ±0.218
HSP70	5.82 ^X ±0.339	3.31 ^Y ±0.208
MnSOD	5.08 ^X ±0.274	2.99 ^Y ±0.197

The values are mean±SE of seven observations on six animals. The values with different superscripts within the same row differed significantly ($P \leq 0.05$).

Table 4. Correlation among biochemical parameters in Karan Fries heifers

	THIavg	SOD	GPx	CAT
THIavg	1			
SOD	0.20**	1		
GPX	0.25**	0.65**	1	
CAT	0.14	0.27**	0.21*	1

**($P < 0.01$), *($P < 0.05$).

greater in thermoneutral environment than under heat stress in poultry. Na and Surh (2008) observed anti-stress effects of phytochemicals through activation of Nrf2/HO-1 pathways. Further, Sahin *et al.* (2010) found negative association of heat stress with transcription factor induction, which resulted in a decrease in the expression of Nrf2, when birds were kept at high ambient temperature. Heat stress suppresses the transcriptional activity of Nrf2 by promoting the localisation of transcription repressors, histone deacetylases with Nrf2/ARE and sequestering coactivators like CREB binding protein (CBP) (Liu *et al.* 2008). During oxidative stress free radicals are generated, which inhibits the upregulation of Nrf2 transcription factor by activating the protein kinase B, lead to upregulation of endogenous enzyme MnSOD and chaperone HSP70 to protect the body from heat stress (Sahin *et al.* 2013). Supplementation of astaxanthin (antioxidant) scavenge the free radicals, binds with cytoskeleton protein and causes dissociation and translocation of Nrf2 from cytoplasm to nucleus, which is essential for activation. In nucleus, Nrf2 binds with ARE and mediates their antioxidative action through induction

of phase II enzymes which leads to downregulation of HSP70 and MnSOD (Mustafi *et al.* 2009). The results of present study were compared with the information available in birds, since very limited information is available in case of ruminant animals.

Heat shock proteins (HSPs) mediate important endogenous protective mechanisms to assist acclimatization to changing environments such as heat, cold and UV (Ruell *et al.* 2009). Under stress conditions, HSPs can be considered an important indicator of the ability of cells to resist damage and adapt to environmental stress (Xu *et al.* 2017). Heat stress induces expression of HSPs to protect cells against thermal injury and to sustain the native configuration of proteins and maintenance of cell survivability (Sottile and Nadin 2017). The present study on heat shock protein 70 (HSP70) genes revealed higher expression in heat stressed control group of growing Karan Fries heifers than treatment group. These HSPs acts as molecular chaperons, safeguards the protein folding in a systematic manner and regulation of apoptosis during stressful physiological conditions. The findings of present study revealed upregulation of HSPs during exposure of heat stress. The results of present study are in accordance to Kumar *et al.* (2016) who also found higher level of HSP70 in control group compared to antioxidant (Zn) supplemented group. This higher level of HSP70 in control group might be due to change in the adaptive and physiological mechanism to cope up with the thermal stress and to attain thermo-tolerance. Sheikh *et al.* (2016) reported higher ($P \leq 0.05$) HSP70 mRNA expression at 42°C than zinc supplemented group in KF cows. HSP70 among all the HSPs is most sensitive and positively correlated with heat stress. HSP70 found in cytosol and nucleus and played an important role in protein folding, maintenance of structural proteins, refolding of denatured proteins. The results of Mishra *et al.* (2010) are in accordance to findings of present study i.e. significantly higher HSP70 expression during heat stress in bovine lymphocytes. Maibam *et al.* (2017) reported significant ($P \leq 0.05$) up-regulation of relative HSP70 and HSP70.2 mRNA expression in skin during winter and summer compared to spring (thermoneutral) in Karan Fries cattle. The magnitude of fold change was significantly ($P \leq 0.05$) higher during summer than winter. All above findings clearly showed that HSPs provides protection to

Table 5. Primer sequence for quantitative real time PCR

Gene	Accession No.	Primer sequence	Annealing temperature (°C)	Fragment size (bp)
GAPDH	NM001034034.2	F-CCAACGTGTCTGTTGTGGATCTGA R-GAGCTTGACAAAGTGGTCGTTGAG	58	218
Nrf2	AB162435.1	F-ACCCAGTCCAACCTTTGTCTGT R-AGCTTTTGCCCGTAGCTCATC	59	185
HSP70	GU183097.1	F-TCATCAACGACGGAGACAAGCCTA R-TTCATCTTGGTCAGCACCATCGAC	58	103
MnSOD	NM174615.2	F-TCCACGTCCATCAGTTTGGAGACA R-TTGTTCATGCTGTACATTGCCAG	60	140

the cell from adverse effects of heat stress. In our study, lower fold increase in expression of HSP70 in treatment group might be due to powerful free radical scavenger action of AX, which might protected cells from oxidative damage (Lee *et al.* 2011), stabilizing cellular proteins and protecting them from heat stress induced denaturation and thus modifying acquired thermotolerance. However, the results of Yin *et al.* (2018) are not in accordance to present study, i.e. higher expression of HSP70 in antioxidant treated groups (betaine and vitamin C and sodium bicarbonate) than control groups, respectively. This difference might be due to levels of heat stress, species of animals, management, feeding conditions etc.

Cells of the body are equipped with efficient dismutation pathways; i.e. Cu/ZnSOD acts in the inter-membrane space and MnSOD acts in the matrix to reduce superoxide to hydrogen peroxide (H_2O_2). ROS generation is triggered by oxidative stress during hyperthermia (Dröge 2002). The unfavourable effects of ROS are counteracted by the antioxidant defense mechanism of the cells which includes various antioxidant enzymes and ROS scavenging molecules. Out of various antioxidant enzymes, MnSOD and Cu/ZnSOD are primarily responsible for the conversion of O_2^- to H_2O_2 , which is degraded by catalase or peroxidases (Turrens 2003). Mustafa *et al.* (2009) reported upregulation of MnSOD and HSP70 genes during heat stress in order to neutralize the adverse effects of reactive oxygen species produced during heat stress. MnSOD and Hsp70 are considered as two important pillars of cellular defense and survival and their expression was lowered by supplementation of antioxidant (N-acetyl cysteine) *in vitro*. The results of present study revealed significantly ($P \leq 0.05$) higher MnSOD expression in control than treatment group of heifers. Namekawa *et al.* (2010) also found lower expression of antioxidant gene (MnSOD) in astaxanthin treated bovine embryos exposed to heat stress. Addition of astaxanthin in semen extender of ram sperm significantly ($P \leq 0.05$) reduced malondialdehyde and reactive oxygen species levels during the 72 h storage period (Fang *et al.* 2015). In our study, lower expression of MnSOD gene might be attributed to antioxidant properties of astaxanthin, providing protection against toxic free reactive oxygen species, which are the stimulus for MnSOD. During summer stress, higher upregulation of MnSOD occurred due to mitogen-activated protein kinase (p38MAPK) mediated activation of the Akt pathway during both chronic as well as acute exposure of oxidative stress.

The results of the present study depicts that expressions of Nrf2, HSP70, MnSOD genes in PBMC, antioxidant enzyme in blood plasma of Karan Fries heifers were strongly affected by summer heat stress. Therefore, HSPs, antioxidant enzymes may be used as biomarkers for assessing protective response of tissue against heat stress in heifers. Lower magnitude of HSP70, MnSOD mRNA gene expression and levels of antioxidant enzymes, and higher upregulation of Nrf2 in supplemented group of Karan Fries heifers during summer indicated that astaxanthin

possess potent free radical scavenging activities and protected the cells from oxidative stress. It may be a centrally important mechanism for better adaptability to heat stress in tropical climate conditions. Understanding such molecular mechanisms related to thermoregulation of crossbred heifers may be helpful in devising procedures to improve health and productive efficiency of heat sensitive breeds in hot climate and predicted climate change in future.

In conclusion, Upregulation of MnSOD and HSP70 levels, two important pillars of cellular defense and survival, is due to the p38MAPK-mediated activation of the Akt pathway during heat stress.

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