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Effect of dietary betaine supplementation on growth performance, immunity and oxidative stress in Karan fries heifers during heat stress

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Abstract
Thermal stress leads to increase in blood supply to the periphery and a compensatory reduction in the blood supply to the gut leading to increase production of endotoxins in the body causing injury to the organ and increase in acute phase protein responses in the body. Therefore, a need arises of an antioxidant that protects the gut integrity. Recently betaine, a trimethyl form of glycine has been found to ameliorate heat stress. The experiment was divided into two parts. Experiment-I (thermoneutral conditions) and Experiment–II (summer season : hot-dry and hot-humid season) were carried out on eighteen female Karan Fries heifers were taken and randomly divided into 3 groups (n = 6) such as control, Treatment I (Betaine supplemented @ 25g/d/animal), Treatment II (Betaine supplemented @ 50g/d/animal) when the average maximum and minimum temperatures were (23.5°C and 9.9°C) and (35.3°C and 22.4°C), respectively. Betaine supplementation resulted in significant (p<0.05) increase in DMI, body weight gain and plasma GH. The mRNA expression of IGF-1 was significantly (P<0.01) higher in Treatment I.When the groups were compared during hot dry, hot humid and thermo-neutral season, expression of interleukins (IL-6 and IL-10) showed opposite pattern and expression of MnSOD showed similar pattern. The messenger RNA (mRNA) and protein expression of IL-6 was observed to be higher (P<0.01) in control group than Treatment I and Treatment II group and expression of IL-10 and MnSOD were observed to be higher (P<0.01) in Treatment II followed by Treatment I as compared to control. Plasma level of Acute Phase proteins (Serum amyloid A and haptoglobin) and Total Antioxidant capacity were significantly (p<0.05) lower in betaine supplemented groups as compared to control whereas plasma level of cortisol did not differ significantly. In conclusion, betaine administration enhances nutrient utilisation and have a dwindling effect on expression of cytokines, suggesting a possible role of this potent antioxidant immunomodulator on heat stress amelioration.

Keywords: Heat stress, Betaine, growth performance, cytokines, immune modulator, antioxidant

1. Introduction
The global change in the environment has drawn the attention of researchers to minimize its adverse effect on animal productivity. When the environmental temperature exceeds the body temperature begins to absorb heat, increasing its body temperature from normal to hyperthermia state, leads to heat stress. The productivity of livestock is adversely affected by extreme climatic conditions as well as with different feeding regimens. Reduction in feed intake and production is the common sign of heat stress and different physiological as well as biochemical parameters are also used as an indicator of heat stress. Proper understanding the relationship among climatic factors and feeding regimens will provide a firm basis to improve the health and welfare of animals. Hyperthermia results in redistribution of blood to the periphery and compensatory reduction in the blood supply to the gut, which damages cells lining the gut, leading to endotoxin production in the body. Endotoxin causes tissue damage and acute–phase immune responses. When blood supply resumes, reactive oxygen species and cytokines are released and cause multiple organ injury. Betaine would impact beneficially at several critical points in the progression of thermally induced tissue damage. These include amelioration of damage to gut and liver tissue, and protection against the effects of endotoxin. Thermo-tolerance in animals during prolonged heat stress is characterized by the immunological response and adaptations associated with acclimatization. It is proposed that administration of betaine to livestock would act as an antioxidant, immune-modulator phytogenic feed additive. The immunological response confers transient thermal tolerance, in part due to the expression of Interleukins (IL's). Cytokines are produced in response to a wide

variety of stressors, including oxidative stress (Smolka *et al*., 2000) [40], hyperthermia (Kregel and Moseley, 1996 and Fehrenbach *et al*., 2001) [16, 9]. Haptoglobin, serum amyloid, Total Antioxidant Capacity and IL-6 and IL-10, MnSOD were found to be the most sensitive to temperature fluctuation and suggested as an important molecular biomarker to heat stress in animals. The duration, intensity and ability to recover from the effect of climatic stress are crucial factors affecting performance ability of farm animal (Khalifa, 2003) [15]. The ability of animal to acclimatize and produce optimally under the specific climate condition signifies the adaptation to a particular environmental niche.

Yang *et al.* (2009) [43] studied the effect of feeding of different levels of betaine (2, 4 and 6 % betaine) on growth in pigs and reported that average daily feed intake and feed conversion ratio (FCR) in 2% fed betaine diet was significantly higher (P<0.05) compared with non-supplemented diets. Hassan *et al.* (2011) reported betaine ameliorated the effects of heat stress on weight gain, immunity and body temperature indices in rabbits. Nofal *et al. (*2015) studied the effect of dietary betaine supplementation on productive, physiological and immunological performance in growing chicks under heat stress condition and observed that final body weight, weight gain, feed conversion values and mortality percentage improved significantly by dietary betaine supplementation during the period from 13 - 16 wks of age

To overcome detrimental effects of the heat stress, the basic strategy is to alter the surrounding environment of the animal by using sheds, fans and evaporative cooling (Bucklin *et al*., 1991) [6]. Owing to the tropical climate of the India, semi-intensive nature of rearing cattle it becomes pertinent to search for novel ways to counteract the adverse effect of the heat stress. The provision of nutritional supplements to ameliorate the effects of heat stress in animals is an attractive method and is potentially easiest option for producers, particularly for animals in heat stress. Dietary supplements such as vitamins, trace elements and minerals were exclusively used to ameliorate the adverse effect of heat stress (Tawfeek *et al*., 2014) [41]. Recently betaine, a trimethyl form of glycine has also been found to ameliorate heat stress in sheep (DiGiacomo *et al*., 2012) [8]. Physiologically, mammals utilize betaine as a methyl donor able to participate in protein and lipid metabolism, or when not catabolized, betaine can be used as an organic cellular osmoprotectant (Fernández *et al*., 1998 and Huang *et al*., 2007) [12]. Addition of betaine (0.3 g/kg) to feeds improves performance of poultry under heat stress (Zulkifli *et al*., 2004) [45]. However, it is not completely understood how animals respond to heat stress and betaine modulate the cytokines expression, oxidative and immunological responses in animals. Therefore, the present experiment was designed to investigate the effect of betaine on growth performance, immunity, oxidative stress and expression dynamics of Interleukins and cytokines during heat stress acclimation in Karan Fries cattle.

2. Material and methods

2.1 Geographical location and climatic condition of study area

National Dairy Reseasrch Institute (NDRI) cattle yard is located in the northern part of karnal city in Haryana, India at 250m above mean sea level, in the Indo-Gangetic plains 29º43" N altitude and longitude 77º 2" E. Temperature goes even up to 46ºC on a single day in summer and falls to around 3º C in winter. The relative humidity (RH) ranges from 30 to 85% at this place. The study was carried out for about (9 months) i.e from March 2017 to November 2017 at National Innovation on Climatic Resilient Agriculture (NICRA) of NDRI, Karnal. Maximum temperature humidity index was recorded during hot dry (HD) (moderate stress) followed by hot humid (HH) season (severe stress) and finally thermo-neutral season (comfortable). Calculation of THI was done according to the formula of National Research Council (1971):

$$THI = (Tdb + Tb) \times 0.72 + 40.6$$

Where THI is temperatuwre Humidity index, Tdb is dry bulb temperature (and Twb is wet- bulb temperature (ºC).

All the climatic variables were recorded twice daily at 8.30 A.M. and 2.30 P.M; however we are showing the average value of each meteorological variable during each season

2.2. Experimental design

Clearance of this experimental study was taken from the Animal Ethics Committee Institute according to the article 13 of the CPCSEA rules, laid down by Government of India. Initially, a total of eighteen healthy Karan Fries heifers (6 in each group) were selected from LRC of NDRI and followed for all seasons. The first group is Control (n=6), the second group is Treatment I (n=6) supplemented with betaine @ 25 gm /d/animal and third group is Treatment II (n=6) supplemented with betaine @ 50 gm /d/animal

2.3 Sampling and Management of the animals

All the cows were housed in open air stalls with asbestos roof. These cattle were fed with ad-libitum green fodder (Maize, Jowar, Berseem and Oat and also a measured amount of concentrates diet (20% crude protein and 70% total digestible nutrient) according to National Research Council (2001) [19] recommendation. Fresh and clean drinking water was provided to them throughout the day. Blood samples were (7ml/animal) drawn from these cattle in sterile heparinised vacutainer tubes from jugular vein puncture at fortnightly interval. Plasma was separated and stored for further analysis at -80ºC. DMI of individual growing Karan Fries heifers were observed by recording the feed offered and feed left at daily basis. The DM content of different feed composition was estimated fortnightly. The DMI of heifers in all the groups were recorded daily and average of fifteen days was considered as DMI of the fortnight. Body weight of growing Karan Fries heifers were recorded at the initial day of the experiment by using electronic weighing machine situated in the NICRA building. Body weight of growing animals was recorded at every fortnight interval. The maximum capacity of the weighing machine was 1500 kg and minimum capacity was 4 kg. The animals were weighed consecutively for 3 days and average was considered as their body weight of that particular interval.

2.4 Quantification of plasma inflammatory interleukins, total antioxidant capacity and cortisol levels.

2.4.1 Plasma pro and anti- inflammatory cytokines (Serum Amyloid A and Haptoglobin) were estimated using bovine specific ELISA test kits according to manufacturers' protocols. The desired optical density (OD) was measured by Tecan Nano Quant ELISA reader (Infinite M 200 PRO, Bio Screen Instruments Pvt, Ltd).

2.4.2 Quantitative sandwich enzyme immunoassay for the determination of Serum Amyloid A
SAA was estimated in plasma samples using "Bovine SAA ELISA Kit" (Catalog No. E0023Bo) supplied by Bioassay Technology, 1713 Junjiang International Building, 218 Ningguo Rd. Yangpu Dist. SH. China. Sensitivity : the minimum detectable dose of SAA was typically 0.1- 40 µg/ml.

2.4.3 Quantitative sandwich enzyme immunoassay for the determination of Haptoglobin
Haptoglobin was estimated in plasma samples using "Bovine HPT ELISA Kit" (Catalog No. E0022Bo) supplied by Bioassay Technology, 1713 Junjiang International Building, 218 Ningguo Rd. Yangpu Dist. SH. China. Sensitivity: the minimum detectable dose of Haptoglobin was typically 3- 900 µg/ml.

2.4.4 Quantitative sandwich enzyme immunoassay for the determination of Total Antioxidant Capacity
TAC was estimated in plasma samples using "TAC Assay Kit" (Lot: BH09A08)) supplied by Bioassay systems, 3191 corporate Place, Hayward, CA 94545, USA. Assay range is in between 0.044- 0.330 and inter assay CV% = 3% (n=20) and intra assay CV%= 3.4% (n=84).

2.4.5 Quantitative sandwich enzyme immunoassay for the determination of Plasma Cortisol
CORT was estimated in plasma samples using "Bovine CORT ELISA Kit" (Catalog No. E0110Bo) supplied by Bioassay Technology, 1713 Junjiang International Building, 218 Ningguo Rd. Yangpu Dist. SH. China. The standard curve range was 0.2µg/ml-90µg/ml and sensitivity range = 0.12 µg/ml.

2.4.6 Quantitative sandwich enzyme immunoassay for the determination of Plasma Growth hormone
Growth was estimated in plasma samples using "Bovine GRH ELISA Kit" (Catalog No. E010Bo) supplied by Bioassay Technology, 1713 Junjiang International Building, 218 Ningguo Rd. Yangpu Dist. SH. China. The standard curve range was 0.2µg/ml-90µg/ml and sensitivity range = 0.12µg/ml

2.5 Expression of inflammatory cytokines (IL-6 and IL-10) and MnSOD in PBMCS of Karan Fries cattle supplemented with betaine during heat stress
From each blood sample, isolation of RNA was carried out for the expression study using the Trizol method as described by Chomcznski and Sacchi (1987) [7]. The RNA was subjected to DNase treatment by DNase 1, RNase-free (Thermo Scientific, USA) according to the manufacturer's protocol. The concentration of the purified RNA was determined by measuring absorbance at 260 nm. The purity of RNA was done using Bio Spec-nano (serial no., A116449; Biotech) and judged on the basis of OD ratio at 260:280 nm to be (1.8-2.0). The integrity of each RNA sample was evaluated by agarose gel electrophoresis using 1.5% of high-quality molecular biology grade agarose (Sigma, USA), Tris –EDTA buffer (0.002 EDTA) with (0.05µg/ml) of ethidium bromide. The presence of two distinct intact bands of 28s and 18s without any smearing indicated good quality and intactness of RNA. After RNA isolation had completed, it was stored at -80ºC. Complementary DNA (cDNA) was prepared from 1µg of RNA, using the Novagen first strand (cDNA) synthesis kit (La Jolla, CA, USA) according to manufacturer's protocol. Synthesised cDNA was kept at -80ºC until use. Primers specific for bovine (IL-6 and IL-10) are presented in table 2. The fragment size of PCR amplified products was confirmed by agarose gel electrophoresis using 1.5% agarose gel against references genes 100bp and 50bp DNA ladders (Fig 1). Real time quantitative reverse transcription PCR (qRTPCR) was performed by Roche's Lightcycler 480 instrument (Pfaffl, 2001) [23] with some modifications. Reaction mix for (qRTPCR) was prepared as follows: 1µg template; 5µl (2X) SYBR green mixes, 0.5µl each of reverse and forward primer, and 3µl nuclease free PCR grade water. The following protocol for the Light Cycler 480 was used: initial denaturation at 95ºC for 5 min, followed by 35 cycles at 95ºC for 30s, at the appropriate annealing temperature for 30s and then 72ºC for 5 min and the final cooling temperature was 4ºC. For normalisation of qPCR data, GAPDH was used. The calculation was done using the 2^{-CT}

2.6 Statistical analysis
Gene expression data were expressed as fold change relative to standard control sample that run within each assay plate. Values were adjusted for GAPDH mRNA expression. They represent the relative fold change compared with the value of control group of all the seasons which was considered as a control for the relative expression study. All the data were analysed by repeated measures two-way ANOVA for between the group (seasons and groups) analysis, and hypothesis testing was done at 5% and 1% significance level. Pairwise comparison of means were tested by Duncan multiple range test (DMRT) comparison test using SAS software, version 9.1 SAS system for window, copyright (2011), SAS Institute Inc., CARY, NC, USA.

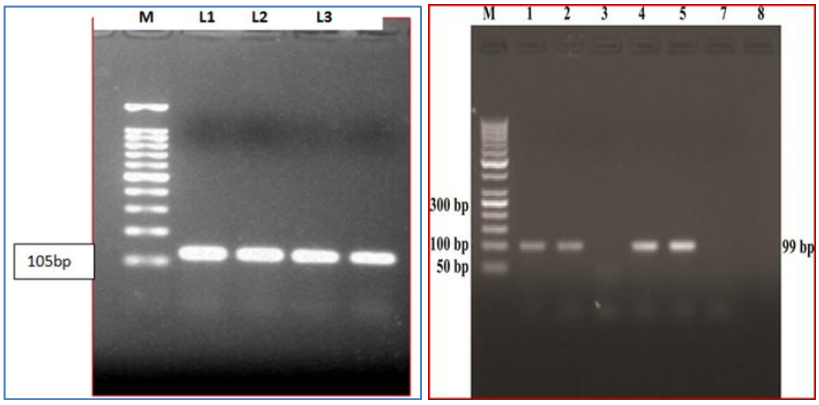

Fig 2: PCR Amplified products of gene on agarose IL-10 (99bp) and IL-6 (105bp)(1.5%) atoptimized annealing temperature and bp =100 ; its melting curve during qRT-PCR

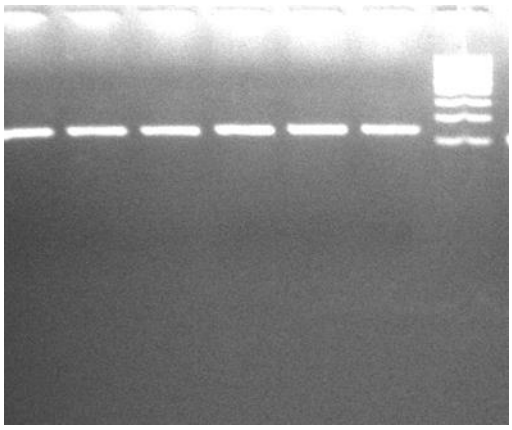

Fig 3: PCR Amplified products of gene MnSOD (140bp) on agarose (1.5%) at optimized annealing temperature and bp =100 ; its melting curve during qRT-PCR

Table 1: Details of various primers used in the study

SL No.	Gene Name	Product Size	Accession No.	Tm (ºC)	Primer Sequence
1	GAPDH	109 bp	XM_006065800.1	58.0	FP: CCAACGTGTCTGTTGTGGATCTGA RP:GAGCTTGACAAAGTGGTCGTTGAG
2	IL-6	105 bp	AY347710.1	59.0	FP: ATGACGAGTGTGAAAGCAGC RP: TCGCCTGATTGAACCCAGAT
3	IL-10	99 bp	AY887900.1	59.0	FP: ATGACGAGTGTGAAAGCAGC RP: TCGCCTGATTGAACCCAGAT
4	MnSOD	140 bp	NM_174615.2	60	FP:TCCACGTCCATCAGTTTTGGAGACA RP:TTGTCATGCTGTCACATTGGGCAGA

Table 2: Values of Temperature Humidity Index (THI) during different seasons and months of study period

Season	Month	Max. temp (°C)	Min. temp (°C)	RH %	Tdb (ºC)	Twb (ºC)	THI
Hot Dry	April	35.00	18.00	25.00	33.20	20.50	79.26
	May	36.10	24.50	31.50	36.80	26.20	85.96
	June	35.55	24.20	23.60	35.20	25.30	84.16
	Mean	35.33	22.23	26.70	35.16	24.00	83.12
Hot Humid	July	33.50	25.30	71.20	30.00	27.50	83.4
	Aug	34.90	27.10	73.00	32.80	28.50	84.73
	Sept	34.20	26.20	72.10	35.20	30.20	86.24
	Mean	34.52	23.40	50.40	31.60	30.40	85.27
Thermo-neutral	Oct	30.30	16.80	43.00	30.25	22.2	78.36
	Nov	27.30	13.10	40.00	22.65	15.25	67.88
	Mean	23.90	9.90	47.00	26.45	18.725	73.12

3. Results

3.1 Effect of betaine supplementation on Acute phase proteins viz Serum amyloid A and haptoglobin

The data presented in Fig 7 indicates that the mean values of Serum amyloid A (ug/ml) in Karan Fries heifers during hot dry season in Control, Treatment I and Treatment II were 198.88±3.72, 165.50±6.50 and 161.91±3.65, respectively. In hot humid season mean values of Serum amyloid A (ug/ml) in Karan Fries heifers in Control, Treatment I and Treatment II were 150.86±2.77, 130.35±4.21 and 139.11±2.63, respectively and during thermo-neutral season mean values of Serum amyloid A (ug/ml) in Control, Treatment I and Treatment II were 123.73±4.81, 110.60±3.40 and 110.46±4.08, respectively. Serum amyloid A (ug/ml) was significantly higher ($p<0·05$) in Control as compared to supplemented groups whereas there was no significant difference between the treatment I and II. All the seasons under study had significant effect ($p<0·05$) on Serum amyloid A (ug/ml) within all the treatment groups.

Mean values of Haptoglobin (ng/ml) of Karan Fries heifers supplemented with different levels of Betaine during three seasons have been presented in Fig 6. In Karan Fries heifers during hot dry season in Control, Treatment I and Treatment II were 237.77±3.89, 219.52±4.49 and 220.33±6.95, respectively. In hot humid season mean values of Haptoglobin (ng/ml) in Karan Fries heifers in Control, Treatment I and Treatment II were 245.30 ± 4.69, 210.14 ± 2.07 and 199.51 ± 5.00, respectively and during thermo neutral season mean values of haptoglobin (ng/ml) were 180.47 ± 5.58, 173.83 ± 3.12 and 130.16 ± 3.00, respectively. There was no significant effect of hot dry and hot humid season treatments on haptoglobin. During Thermo-neutral season mean values of haptoglobin were significantly similar($p<0·05$) in treatment I and control as compared to Treatment II.

Total antioxidant Capacity (mM/dl) of Karan Fries heifers supplemented with 25 and 50g/d/animal of betaine during three seasons have been depicted in (Fig 4). Mean values of Total antioxidant Capacity in Karan Fries heifers during hot dry, hot humid and thermo neutral season in Control, Treatment I and Treatment II were 1.85 ± 0.23, 2.11 ± 0.15 and 3.25 ± 0.40 mM/dl, respectively 0.90 ± 0.29, 2.45 ± 0.25 and 3.75 ± 0.48, and 1.05 ± 0.28, 2.00 ± 0.38 and 3.45 ± 0.51, respectively. Statistical analysis revealed significant difference ($p<0.01$) between treatment groups. Higher TAC

activity was seen in Treatment II as compared to Treatment I over control. There was no significant effect of hot dry and hot humid season on mean values of Total Antioxidant capacity between groups.

Mean values of Cortisol (ng/ml) of Karan Fries heifers supplemented with different levels of betaine during different seasons have been presented in Fig 5 in hot dry season in Control, Treatment I and Treatment II were 10.86 ± 0.22, 9.93 ± 0.35 and 9.74 ± 0.25, respectively. In hot humid season mean values of Cortisol (ng/ml) Karan Fries heifers in Control, Treatment I and Treatment II were 10.36±0.16, 9.07±0.27 and 9.82±0.25 respectively and during thermo neutral season mean values of Cortisol (ng/ml) in Control, Treatment I and Treatment II were 5.77±0.35, 4.34±0.27 and 4.29±0.10 respectively. There was no significant (p>0.05) effect of betaine supplementation on mean values of Cortisol (ng/ml) in the groups, whereas all hot dry and hot humid season had no significant (p>0·05) effect on mean values of Cortisol (ng/ml) within the treatments and control group, similar findings were reported by Raheja (2017) who also observed non-significant difference in cortisol level on day of calving, although values were numerically less in betaine supplemented groups

3.2 Effect of betaine supplementation on expression of cytokines viz IL-6 and IL-10

The results of mRNA expression IL-6 and IL-10 in PBMC of Karan Fries heifers supplemented with different levels of betaine during three seasons have been presented in Fig 8 and Fig 9, respectively

The expression of IL-6 declined significantly (p<0.01) in Treatment II followed by Treatment I in all the seasons. The mean quantity of relative IL-6 mRNA expression in lymphocyte in Karan Fries heifers during hot dry, hot humid and thermo-neutral seasons were (1.160.04 and 1.25±0.06), (1.33±0.03 and 1.51±0.03) and (1.75±0.02 and 1.92±0.06) fold change in Treatment I and Treatment II respectively. The fold change expressions were reduced in Treatment I by (20%, 25% and 43 % and in Treatment II by (14%,34%and 48%) during hot dry, hot humid and thermo neutral season respectively.

The result of IL-10 in PBMC of Karan Fries cattle have been presented in fig 9, the increase in IL-10 gene expression (1.61±0.044 and 1.78±0.05) fold change during hot dry condition. Further, the values were significantly (p<0.01) higher in hot humid condition (2.52±0.21 and 2.63±0.07 fold) in Treatment I and Teatment II respectively. Then, declines in values were observed in thermo-neutral condition (1.86±0.14 and 1.87) fold. Statistical analysis revealed a significant difference (p<0.01) in Treatment groups in hot dry and hot humid condition a linear increase in IL-10 activity was observed with betaine supplementation. Thus, Treatment II showed greater increase in IL-10 and IL- 6 activities followed by Treatment I. However, no significant difference in supplementation rate was observed during Thermo-neutral season.

The results relative expression of MnSOD have been presented in Fig 10.The relative mRNA expression of MnSOD was significantly (p<0.01) higher in Treatment groups. The increase in expressions were (1.90 fold,1.92 fold) and (1.98, 2.04 fold) in treatment I and treatment II during hot dry and hot humid season respectively. In Thermo-neutral season a decline in expression was observed in Treatment groups as compared to control (0.82 and 0.81 fold). However there was no difference within the treatments.

3.3 Body weight, growth performance, dry matter intake and feed conversion efficiency

Mean values of final body weight change in Karan Fries heifers during the study period in Control, Treatment I and Treatment II were 221.13±1.8, 237.16±1.20 and 243.46±1.3, respectively. Average Daily Weight gain (g/d) during feeding trial was 376.7±0.811, 442.4±2.63, and 446.2±1.98g/d/animal in Control, Treatment I and Treatment II, respectively. Average dry matter intake (kg/day) 5.920±0.13 6.210±0.09 and 6.280±0.17 in Control, Treatment I and Treatment II, respectively, Dry matter intake and Average Daily Gain in betaine supplemented group i.e Treatment I and Treatment II were significantly higher as compared to Control whereas there was no significant difference between Treatment I and Treatment II. However, dry matter intake and average daily gain in Treatment I was numerically higher as compared to Treatment II. Feed conversion efficiency (DMI/ Weight gain) was similar between the treatment groups (0.138,0.139 vs 0.157 %) and were significantly higher in Treatment I and Treatment II as compared to Control indicating supplemented groups require less expenses of energy (DMI) to produce desired output (Weight gain). Studies in grower and finisher pigs indicated that dietary betaine reduces heat production and energy requirements for maintenance, effectively increased energy retention (Sharma *et al*. 2013) [25]. Our findings are in agreement with Zulkifli *et al.* (2004) [45] and Nofal *et al.(*2015) who also reported that final body weight, weight gain and feed conversion values improved significantly by dietary betaine supplementation in growing chicks under heat stress condition.

4. Discussion

The present study was carried out to see the effect of dietary betaine supplementation on immunological responses of karan fries cattle during heat stress. Various environmental parameters were recorded, and THI measured as it is a valuable indicator that can be effectively used to assess the effect of environmental stress on dairy cattle. When THI values goes beyond 72 units, the zone of heat stress starts, and animal usually triggers various physiological and immunological mechanisms to maintain homeothermy and protect its body against the invading pathogens (Zhang *et al*., 2014) [44].

Serum amyloid A and Haptoglobin were estimated from plasma samples as they are indicator of acute phase protein responses. SAA is a high-density lipoprotein (HDL) and a major Acute Phase Protein in many animal species including ruminants. Higher levels of Bovine SAA in control clearly indicates stress in the animals as during heat stress, there is increase in blood supply to the periphery, leading to increase production of endotoxin and increase production of acute phase proteins. There was decline in SAA in Treatment I and Treatment II because of reduction in effect of stress due to supplementation of betaine in the diet @ 25 and 50g per day per animal this may be attributed to anti-apoptic property of betaine. Haptoglobin is a major Acute Phase Protein (APP) in ruminants. During periods of starvation, there is an elevation in Acute Phase Protein in cows (Katoh and Nakagawa, 1999) [18] and in cattle during summer due to production of pro-inflammatory cytokines (Vels *et al.* 2009) [42]. High relative increase in cattle during an acute phase response, were suggestive of biomarkers of inflammation or health (Huzzey *et al.* 2011) [13]. Significant (p<0.05) decline in haptoglobin activity in treatment I and Treatment II (8% to 16%) in hot

dry and humid condition respectively as compared to control indicates betaine supplementation was affective in modulating the immune system by reducing the adverse effect of heat stress. Gurjar *et al.* (2016) [11] reported a rise in haptoglobin level in KF heifers on 3 hours of exposure in climatic chamber (40 and 42ºC and 50% humidity). The findings can be correlated with lower levels of IL-6 in betaine supplemented groups.

TAC is considered as an cumulative action of all antioxidant present in plasma and body fluids It describe the dynamic equilibrium between pro oxidant and anti oxidant agent in the plasma compartment. Control group heifers exhibited marked reduction in plasma level of antioxidant as indicated in the current data. Thermal stress leads to oxidative stress in animals 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 (Patir 2010) [22]. O'Brien *et al.* (2009) [20] also found reduction in Total Antioxidant Capacity (TAC) level during estrus cycle in heifers during hot season. As TAC helpful to measure stress in ruminant increase level of TAC in Treatment I and Treatment II indicates positive effect of betaine supplementation in ameliorating heat stress. Increase TAC level in supplemented groups can be positively correlated with increase in SOD, catalase and decrease in MDA levels in betaine supplemented groups (Table 4.33). The ability of betaine to protect against oxidative stress is attributed to the fact that betaine is highly lipotropic and,when administered exogenously, it can readily pass across the membrane lipid bilayer and diffuse into intracellular compartments (Kanabak *et al*. 2001) [14]

Higher cortisol levels during hot dry and hot humid season indicates thermal stress to the animals and could be due to the activation of hypothalamic-pituitary-adrenal axis and consequent increase in plasma glucocorticoids (Marai *et al*. 2007 and Marie *et al.* 2001). These findings were in agreement with the Megahed *et al*. (2008) in buffalo cows, Kumar *et al*. (2010) in buffaloes, Sivakumar *et al.* (2010) [39] in Black Bengal goats, Bhan *et al*. (2013) [5] in growing and adult Sahiwal cattle, Sharma *et al.* (2013) [28] in goats, Sejian *et al*. (2013) [24] in Malpura ewes and Al-Samawi *et al*. (2014) [2] in Ardi-goats. Zhang *et al.* (2014) [44] showed that the supplementation of 15 g / day of betaine was the most favorable quantity for stabilizing the endocrine hormone content of cows under the heat stress.

IL-6 is proinflammatory cytokines which are upregulated during thermal stress in control group as they are the biomarkers of stress and inflammation. Down regulation of IL-6 expression in betaine supplemented indicates a relief from heat stress as betaine plays a role in alleviating heat stress, they prevent apoptosis further providing better immunity as in the study. Parker *et al*, (2004), observed that heat shock protein increase synthesis of and induction of IL-6 in culture media, reduction in HSP production was observed on betaine supplementation in our study, this could be correlated with less production of IL-6. IL-6 and IL-10 are cytokines with opposite effects where IL-6 promotes cell death and IL-10 promotes cell survival following apoptic stimuli. In summer season IL-10 expression was found to be higher in supplemented groups as compared to control. Betaine activity as an immuno modulator has been described in broilers (Kim and Kim 2002). Betaine supplemented @ 200 mg/kg had shown positive effects regarding mortality reduction under heat stress conditions, improved immunity, and the health performance in birds (Attia *et al.* 2016) [3].

To the best of our knowledge, we are the first to report the effect of betaine supplementation on mRNA expression of IL-6 and IL-10 genes in Karan Fries heifers during heat stress.

The present study indicated that MnSOD gene induction takes place due to heat stress. The increase in activity of MnSOD in treatment groups reflects an attempt to diminish the superoxide radical challenge. In the present study relative expression was highest during hot-humid season. It may be due to high degree of thermal stress, which is a part due to cellular events taking place extensively, SOD enzyme expressions seem to play important roles in protection of cells against thermal stress.

Upregulation of MnSOD activity in betaine supplemented groups may be because of betaine which posses potent antioxidant property, it enhances MnSOD free radical scavenger property. Also MnSOD expression is negatively correlated with HSP. The results are in agreement with Sauer, (2003) who reported lower expression of HSP 72 found to upregulate both Mn-SOD and Cu,Zn-SOD mRNA in response to oxidative stress. Zhang *et al*. (2014) [44] evaluated effect of betaine in reducing heat stress in HF cows and observed an increase in GSH and SOD activity in cows supplemented with betaine.

Betaine supplementation improves nutrient digestibility due to its osmoprotective properties, supporting intestinal cells and the growth of intestinal microbes. Average DMI was significantly (p<0.05) higher in betaine supplemented group as compared to control. The results are in agreement with (Ratriyanto *et al*. 2009) who observed an improvement in CF digestibility in poultry indicating that betaine has the potential to stimulate the bacterial fermentation of dietary fibre. These results confirm previous observation that intestinal bacteria require compatible osmolytes, such as betaine.

5. Conclusions

Our findings indicates that betaine at the rate 25g/d/animal act as a potent immunomodulator, it enhances immunity in karan fries cattle and improves growth performance during heat stress.

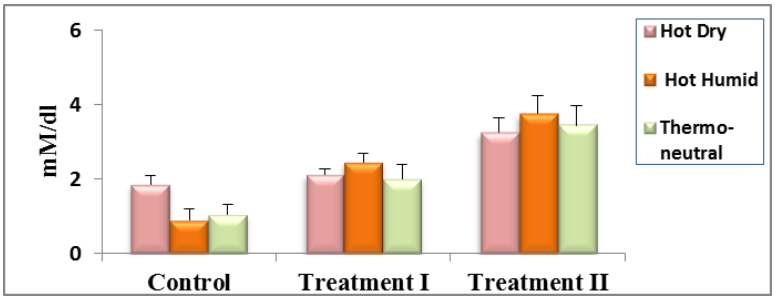

Fig 4: Mean values of Total antioxidant Capacity (mM/dl) in control and supplemented groups during study period

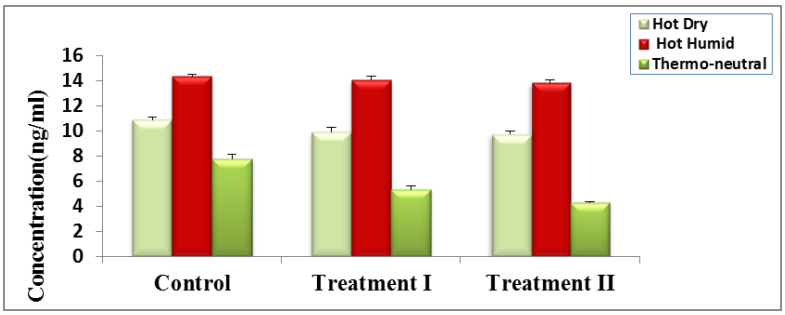

Fig 5: Mean values of Cortisol (ng/ml) in control and supplemented groups during study period

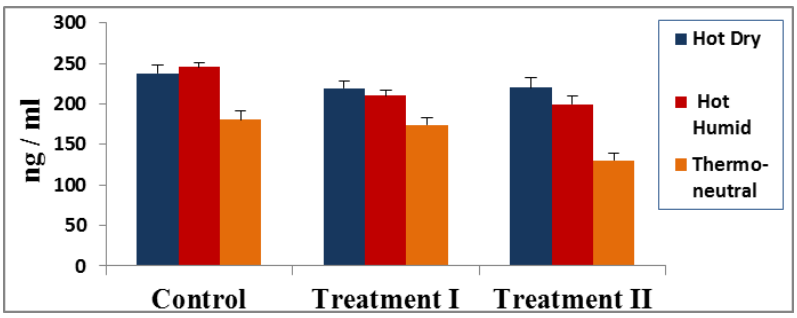

Fig 6: Mean values of Haptoglobin (ng/ml) in control and supplemented groups during study period

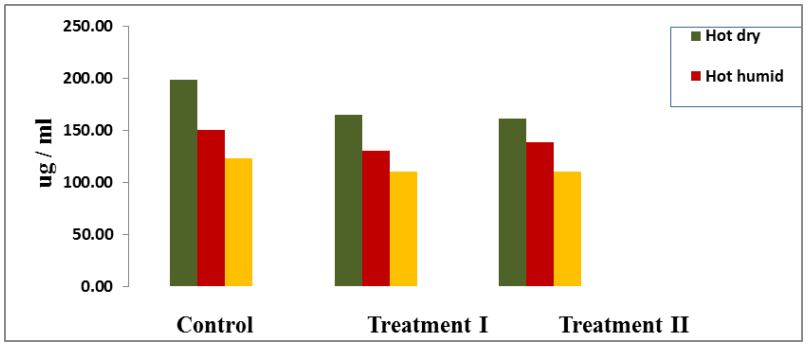

Fig 7: Mean values of Serum amyloid A (ug/ml) in control and supplemented groups during study period

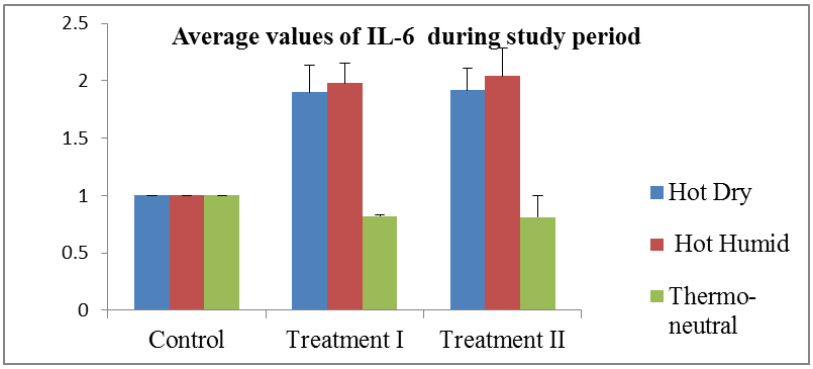

Fig 8: Fold change in mRNA expression of IL-6 in control and supplemented groups during the study period

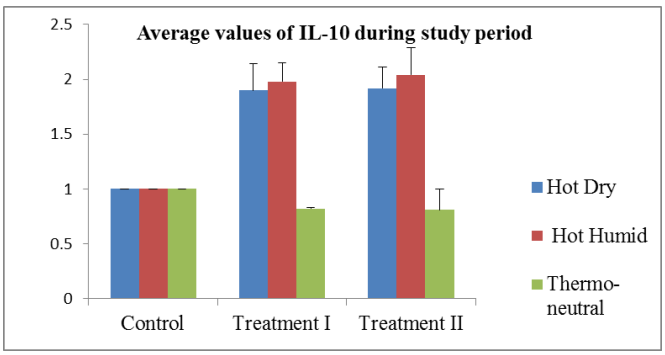

Fig 9: Fold change in mRNA expression of IL10 in control and supplemented groups during study period

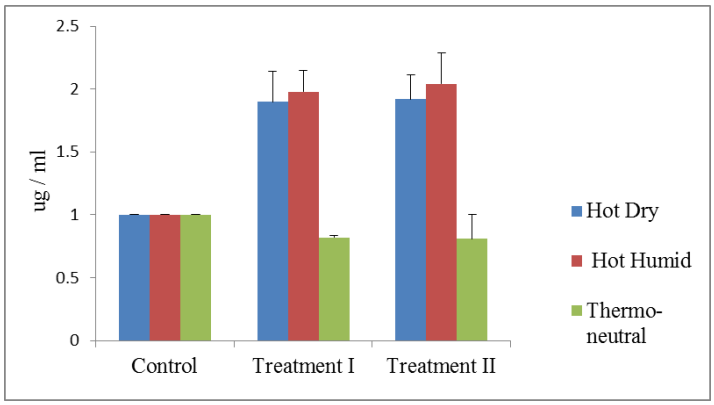

Fig 10: Fold change in mRNA expression of MnSOD in control and supplemented groups during study period

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