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Role of Ascorbic Acid in Modulating Stress Responses and *HSP* Gene Expression in Kachhi Sheep under Heat Stress

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Dietary interventions, Heat stress, Kachhi sheep, HSP-70 and 90, Physiological response, Oxidative stress

ABSTRACT

This study evaluated the effects of ascorbic acid (AA) supplementation on heat stress in Kachhi sheep under extreme temperature, addressing the limited research on protecting sheep from extreme heat, such as the 50–54°C recorded in Sindh province during summer. Fifteen lambs were divided into three groups: KC (control, 35–45°C), KPC (positive control, 30–35°C), and KAA (AA-supplemented, 2ml/day/lamb, 35–45°C). The experiment lasted 90 days. Quantitative PCR revealed upregulated HSP-70 and HSP-90 expression in KC lambs, with significant downregulation in KAA and KPC. Results showed KC lambs had significantly higher physiological stress indicators, while KAA and KPC lambs exhibited lower stress. Hematological analysis indicated increased RBCs and Hb in KAA lambs, with reduced white blood cell counts, with no significant changes in other hematological parameters among groups. Hormonal analysis showed higher T3 and lower cortisol in KAA lambs, with no significant changes in thyroxine. Antioxidant evaluation revealed significantly higher superoxide dismutase in KAA lambs, while glutathione peroxidase and malondialdehyde showed no significant differences. Serum biochemical analysis indicated higher glucose in KPC lambs, while total protein, potassium, and magnesium were significantly higher in KAA lambs. Aspartate aminotransferase levels were highest in KC, with no significant differences in sodium, calcium, ALT, creatinine, or urea nitrogen. Overall, AA supplementation improved heat tolerance by reducing heat shock protein expression and enhancing physiological, hematological, hormonal, oxidative stress, and biochemical parameters, supporting better adaptation to hot climates. These findings highlight AA's potential to mitigate heat stress in sheep under extreme conditions.

INTRODUCTION

Heat stress is a major challenge in livestock production, particularly in regions with high ambient temperatures, such as arid and semi-arid areas (El-Tarabany *et al*., 2017). It occurs when the heat load surpasses an animal's ability to dissipate excess heat, leading to physiological and metabolic disturbances that negatively affect growth, reproduction, and overall productivity (Dikmen *et al*., 2008; Collier *et al*., 2017). Sheep, like other mammals, rely on a combination of behavioral, physiological, and biochemical mechanisms to maintain thermal balance, but prolonged exposure to heat stress can impair their adaptive capacity (Silanikove, 2000). Their thermoneutral zone ranges from 12°C to 32°C, with significant variations in the temperature-humidity index (THI) influencing their ability to cope with heat stress (Shelton, 2000).

Heat shock proteins (HSPs), particularly HSP-70 and HSP-90, play a crucial role in cellular adaptation to thermal stress. HSP-70 prevents protein denaturation, assists in protein folding, and enhances thermotolerance, while HSP-90 prevents irreversible protein aggregation during heat stress (Chen *et al*., 2006; Sharma *et al*., 2013). Increased extracellular HSP-70 level have been observed in heat-stressed animals, underscoring their importance in stress regulation (Min *et al*., 2015).

Several physiological indicators reflect heat stress in sheep, including elevated body temperature, increased respiration rate, and altered hematological and biochemical parameters (Srikandakumar *et al*., 2003; Marai *et al*.,

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2007). Blood profile changes, such as reductions in red blood cell count (RBCs), hemoglobin (Hb) concentration, and packed cell volume (PCV), occur due to oxidative damage, increased oxygen demand, and decreased nutrient intake (Temizel *et al*., 2009; Sivakumar *et al*., 2010). Furthermore, heat stress suppresses thyroid hormone secretion (Nazifi *et al*., 2003; Rasooli *et al*., 2004), while elevating cortisol levels were to facilitate physiological adaptations (Indus and Pareek, 2015). Oxidative stress, characterized by excessive reactive oxygen species (ROS) production, exacerbates cellular damage unless neutralized by antioxidant defense systems such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) (Halliwell and Gutteridge, 2015; Ayemele *et al*., 2021). Heat stress also disrupts electrolyte balance, induces hyperglycemia via cortisol-mediated gluconeogenesis, and affects liver and kidney function, as indicated by elevated liver enzymes and blood urea nitrogen (West, 1999; Bernabucci *et al*., 2002).

Among the various strategies to mitigate heat stress, nutritional interventions, including antioxidant supplementation, have gained attention. Ascorbic acid (vitamin C), a potent antioxidant, is essential for immune function and cellular defense against oxidative stress (McDowell, 2000). While ruminants can synthesize AA endogenously, its concentration declines under stress conditions, necessitating dietary supplementation to support health and productivity (Ranjan *et al*., 2012). Despite extensive research on heat stress and its impact on livestock, limited information is available on the effects of AA supplementation on heat shock protein expression and physiological responses in sheep. Therefore, the objective of the present study was to evaluate the effects of AA supplementation on *HSP-70* and *HSP-90* gene expression, as well as physiological, hematological, hormonal, and oxidative stress responses in Kachhi sheep exposed to hot climatic conditions.

MATERIALS AND METHODS

Experimental animals and their management

The study was conducted from May to July at Sindh Agriculture University Tandojam, Department of Livestock Management, located 29 meters above sea level in a semi-arid region (25°25'37.85" N, 68°32'10.28" E). Fifteen male Kachhi lambs (3-4 months old) were purchased from local markets in Hyderabad and surrounding districts of Sindh. They were housed in well-ventilated sheds with free access to fresh water. All lambs received a basal diet of 70% roughage and 30% concentrates (Table I).

Experimental design

The experimental trial was conducted for 90 days, including a 15-day adaptation period, with lambs randomly divided into three groups (n=5) based on body weight. The lambs had an average starting body weight of about 11.67±1.82 kg. The body weights were very similar across the groups, with non-significant difference (p= 0.873). Group 1 (the Kachhi Control, KC), was kept intensively at the Livestock Experiment Station under an average temperature of 35-45°C. Group 2 (Kachhi Positive Control, KPC) lambs were maintained at 30-35°C using an air cooler, while Group 3 (Kachhi AA supplemented KAA) received 2ml/day/lamb of Vitalite C12 (Innovad which contains 250 mg active concentration of AA and were kept under similar conditions as KC. The environmental conditions were monitored with the help of automatic humidity temperature clock (HTC-1). The average temperature-humidity index (THI) values were determined by daily records of ambient temperature and relative humidity (RH) using the formula as proposed by Marai *et al*. (2001).

Expression of HSP *genes*

Blood samples were collected via jugular venipuncture into EDTA tubes for hematological analysis and plain tubes for serum biochemical and hormonal assays. Serum was separated by centrifugation (3000 rpm for 15 min) and stored at -20°C until analysis.

The expression of HSP-70 and HSP-90 genes was determined using qRT-PCR, with RNA extracted via the Sloarbio total RNA extraction kit (R1200), quantified using a NanoDrop 2000 spectrophotometer, and reverse transcribed into cDNA using a 1-step Solis Green kit (SOLIScript). The primers used for qRT-PCR assay were based on previous sequence information from the National Center for Biotechnology Information (NCBI), targeting *HSP-70* and *HSP-90* genes as described by Younis (2020)

Table I. Ingredients and chemical composition of basal ration.

Ingredients	Moisture (%)	Dry matter (%)	Crude protein (%)	Crude fiber (%)	Ether extract (%)
Lucerne (*Medicago sativa)*	78 ± 3	22 ± 3	18 ± 1.5	27 ± 2.5	2.5 ± 0.3
Wheat straw (*Triticum aestivum* -residue)	10 ± 1	90 ± 1	3.5 ± 0.5	37 ± 2	1 ± 0.2
Wheat bran (*Triticum aestivum* by-product)	12 ± 2	88 ± 2	15 ± 2	11 ± 1	4 ± 0.5

Table II. Primers used in this study.

Desired gene	Sequence (5′ →3′)	Accession No
HSP-70	F = GACAAGTCGGAGAACGTGCA	JN604434
	R = CGTACACCTGGATCAGCAC	
HSP-90	F = ATTGACATCATCCCGAATC	EF091713
	R =ACACCAAACTGCCCAATCAT	
GAPDH	F = GCAAGTTCCACGGCACAGTC	AF030943
	R =CCCACTTGATGTTGGCAGGA	

HSP70, heat shock protein 70; HSP90, heat shock protein 90; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

(Table II). All reactions were estimated in duplicate to ensure the reliability of the results. A total reaction volume of 20 µl was used for amplification, consisting of 1 µl of forward primer, 1 µl of reverse primer, 2 µl of RNA template, 10 µl of 2× PCR master mix, and 6 µl of nuclease-free water. The qRT-PCR reaction conditions comprised initial denaturation at 95°C for 10 min, followed by 45 cycles, each of extension at 95 °C for 10 sec, annealing at 53°C for 30 sec, extension at 72°C for 30 sec, and cooled at 60 °C for one min. PCR amplification was verified using 1% agarose gel electrophoresis and post-PCR melt curve analysis. GAPDH was used as the endogenous reference gene for normalization of target gene expression. Quantitative real-time PCR (qRT-PCR) was performed using the AB applied biosystems 7300 real-time PCR system. Relative gene expression was calculated using the 2-ΔΔCT method, as described by Livak and Schmittgen (2001). The threshold cycle (Ct) values were obtained for both the target and reference genes, and the ΔCt was determined by subtracting the Ct of GAPDH from the Ct of the target gene. The ΔΔCt was then calculated by comparing the ΔCt of the treatment group with that of the control group. The fold change was calculated as 2−ΔΔCt. Fold change data were statistically analyzed using one-way ANOVA in JMP 7 software. Post hoc comparisons were performed using Tukey's HSD test where applicable. Results are expressed as mean ± standard error of the mean (SEM), with significance set at $p<0.05$.

Physiological parameters were recorded following Reece *et al*. (2015).

Hematological parameters, including RBC count, hemoglobin concentration (Hb), packed cell volume (PCV), WBC count, MCV, MCH, and MCHC, were analyzed using a Nihon Kohden hematology analyzer (Schalm *et al*., 1975).

Hormonal concentrations of triiodothyronine (T3), thyroxine (T4), and cortisol were measured via Electrochemiluminescence Immunoassay (ECLIA) using a Cobas e immunoassay analyzer. The assays had detection sensitivities of 0.3 ng/mL for T3, 0.5 µg/dL for T4, and 1.5 nmol/L for cortisol. The intra-assay coefficients of variation (CVs) were <5% and inter-assay CVs were <8% for all three hormones, indicating acceptable analytical precision. All assays were performed according to the manufacturer's instructions.

Oxidative stress markers, including SOD, GSH-Px and MDA, were analyzed using assay kits from Nanjing Jincheng Bioengineering Institute (China). All procedures strictly followed the manufacturers' protocols to ensure accuracy and reliability

Serum biochemical parameters such as glucose, total protein, sodium, potassium, calcium, magnesium, urea nitrogen, creatinine, and liver enzymes (ALT and AST) were assessed using a Roche Hitachi C311 automatic analyzer (Alhidary *et al*., 2012).

Statistical analysis

Statistical analysis was performed using JMP software, version 7.0, with the significance level set at ($p<0.05$). The relative expression levels of HSP-70 and HSP-90 mRNA, normalized to the housekeeping gene GAPDH, were analyzed using one-way ANOVA. The means for each group were compared using Tukey's HSD post-hoc test.

RESULTS

Gene expression analysis of HSP-70 and 90 genes

qRT-PCR analysis showed unchanged HSP-70 and HSP-90 expression in the KC group (fold change: 1.00) but downregulation in KPC (0.51 and 0.75) and KAA (0.36 and 0.39) groups (Table III). Melt curve analysis and agarose gel electrophoresis confirmed this pattern, showing stronger HSP-70 and HSP-90 bands in KC, with reduced expression in KPC and KAA (Fig. 1).

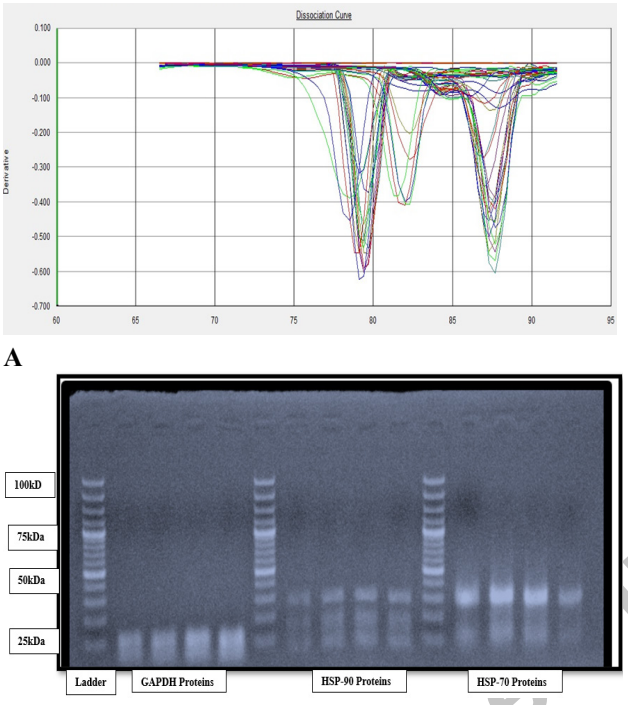
Physiological responses

The results of the physiological responses are shown in Table IV. It was noted that rectal temperature, pulse rate, and respiration rate were significantly elevated ($p<0.05$) in intensively kept KC lambs and reduced in KPC maintained between (30 to 35°C) and KAA lambs supplemented with AA.

Hematological parameters

Hematological parameters were measured on days 0 (before treatment) and 90 (after treatment) of the experimental trial. The study revealed a significant ($p < 0.05$) difference in RBC and Hb concentrations, with KAA lambs exhibiting the highest level, followed by KPC lambs, and the lowest level observed in KC lambs. In contrast, PCV remained unaffected ($p > 0.05$) across

all groups. Furthermore, AA supplementation significantly ($p < 0.05$) reduced WBC counts in KAA lambs compared to KC and KPC lambs, whereas WBC counts increased. Additionally, no significant ($p > 0.05$) differences were observed in MCV, MCH, and MCHC among all groups (Table IV).



A

B

Fig. 1. Melt curve analysis (A) and agarose gel electrophoresis (B) of qRT-PCR amplification products for target and reference genes.

Hormonal and oxidative stress parameters

Hormonal analysis revealed the highest T3 concentration in KAA lambs and the lowest in KC lambs, with no significant differences ($p>0.05$) in the level of T4 hormone among the groups (Fig. 2). Cortisol level were significantly ($p<0.05$) higher in KC and lower in KAA, followed by the KPC group (Fig. 2C). The results of oxidative stress parameters indicated that the level of superoxide dismutase was significantly ($p<0.05$) higher in KAA lambs, followed by KPC and lower in KC lambs. However, no significant ($p>0.05$) difference were observed in the concentrations of gGPX and MDA among the groups (Fig. 3).

Serum biochemical parameters

Serum biochemical parameters were evaluated on day 0 and day 90th the experimental trial. The study revealed significant ($p < 0.05$) difference in glucose level, with the highest level in KPC lambs, followed by KAA, and the lowest in KC lambs. Total protein level also improved significantly ($p < 0.05$), with KAA lambs showing the highest values, followed by KPC and KC lambs. Potassium and magnesium levels increased significantly ($p < 0.05$) in KAA lambs, followed by KPC, and decreased in KC lambs. In contrast, sodium and calcium levels were non-significant ($p > 0.05$) across all groups before and after treatment, though higher calcium level were observed in KAA lambs. Non-significant ($p > 0.05$) difference were found in ALT, creatinine, and urea nitrogen level across groups. However, AST level showed a significant ($p<0.05$) difference, with the highest level in KC lambs, followed by KPC, and the lowest in KAA lambs (Table IV).

Table III. Effect of ascorbic acid on expression of *HSP-70* and *HSP-90* genes in Kachhi sheep under heat stress.

Parameter	KC	KPC	KAA	p-value
HSP-70 (average Ct)	38.46 ± 0.05	39.49 ± 1.9	39.58 ± 0.48	-
GAPDH (average Ct)	38.41 ± 0.35	38.46 ± 0.31	38.07 ± 0.47	-
ΔCt$^{\circ}$	0.04 ± 0.002	1.02 ± 1.6	1.5 ± 0.006	-
ΔΔCt$^{\#}$	0.00 ± 0.002	0.97 ± 0.01	1.46 ± 0.21	-
Fold change	1.00^{a}	0.51^{b}	0.36^{c}	<0.0001
HSP-90 (average Ct)	38.04 ± 0.30	38.51 ± 0.39	38.04 ± 0.47	-
GAPDH (average Ct)	39.40 ± 1.02	39.45 ± 1.01	38.07 ± 0.47	-
ΔCt$^{\circ}$	1.36 ± 0.72	0.93 ± 0.62	0.02 ± 0.00	-
ΔΔCt$^{\#}$	0.00 ± 0.72	0.42 ± 0.00	1.33 ± 0.10	-
Fold change	1.00^{a}	0.75^{b}	0.39^{c}	<0.0001

KC, Kachhi sheep; KPC, Kachhi positive control, KAA, Kachhi receiving 2 ml vitalite C12/day/lamb.
For other abbreviations, see Table II.

Table IV. Effects of ascorbic acid on physiological, hematological and serum biochemical parameters in Kachhi sheep under stress as influenced by different management systems.

Parameter		KC	KPC	KAA	SE	p-Value
Physiological parameters						
Rectal temperature (°F)		103.6 ± 0.54[a]	102.4 ± 0.54[b]	102.6 ± 0.89 [ab]	0.305	0.036
Pulse rate (beat/ min)		78.8 ± 1.64 [a]	74.6 ± 3.43 [b]	76.2 ± 0.83 [ab]	1.006	0.036
Respiration rate (breath/min)		30.60 ± 2.07 [a]	25.60 ± 2.19[b]	28.60 ± 3.20 [ab]	1.137	0.027
Hematological parameters						
Red blood cells (10^6/µL)	BT	10.78 ± 1.10	10.50 ± 1.33	10.61 ± 0.85	0.498	0.927
	AT	10.92 ± 0.92[b]	11.52 ± 0.71[ab]	12.28 ± 0.54 [a]	0.332	0.041
Hemoglobin (Hb g/dl)	BT	9.94 ± 1.16	9.54 ± 0.6	9.802 ± 0.51	0.367	0.741
	AT	10.16 ± 0.98 [b]	11.22 ± 0.42[ab]	11.66 ± 0.82[a]	0.315	0.020
Packed cell volume (%)	BT	30.2±3.27	28.8 ± 2.16	29.6 ± 2.07	1.146	0.694
	AT	31.6 ± 2.88	32.2 ± 2.58	33.6 ± 3.20	1.298	0.552
White blood cells (10^3/ µL)	BT	8.86 ± 0.95	8.8 ± 0.56	8.56 ± 0.59	0.367	0.79
	AT	9.50 ± 0.63[a]	9.22 ± 0.62 [ab]	8.49 ± 0.47 [b]	0.259	0.047
Mean cell volume (fL)	BT	26.52 ± 4.29	27.81 ± 4.33	28.11 ± 3.22	1.781	0.802
	AT	28.95 ± 1.57	28.02 ± 2.62	27.41 ± 3.05	1.116	0.629
Mean cell hemoglobin (pg)	BT	9.22 ± 0.55	9.16 ± 1.01	9.28 ± 0.98	0.392	0.976
	AT	9.30 ± 0.66	9.77 ± 0.80	9.5 ± 0.62	0.313	0.584
Mean cell hemoglobin concentration (g/dL)	BT	32.91 ± 1.87	33.27 ± 3.28	33.17 ± 1.36	1.037	0.968
	AT	32.16 ± 1.59	34.96 ± 2.14	34.83 ± 2.53	0.950	0.102
Serum biochemical parameters						
Glucose (mg/dL)	BT	52.6 ± 5.85	50.2 ± 3.27	51.2 ± 4.49	2.084	0.722
	AT	53.8 ± 6.01 [b]	62.2 ± 2.94[a]	58.2 ± 4.14 [ab]	2.034	0.039
Total protein (g/dL)	BT	6.14 ± 0.49	6.18 ± 0.30	6.06 ± 0.55	0.207	0.917
	AT	6.3 ± 0.38[b]	6.82 ± 0.31[ab]	6.98 ± 0.46[a]	0.174	0.043
Sodium (mEq/L)	BT	140.8 ± 3.27	139.6 ± 2.30	139.2 ± 3.11	1.270	0.660
	AT	142.6 ± 2.88	141.2 ± 2.16	140.6 ± 3.64	1.324	0.564
Potassium (mEq/L)	BT	4.06 ± 0.20	4.22 ± 0.31	4 ± 0.33	0.130	0.488
	AT	3.94 ± 0.27[b]	4.42 ± 0.32[ab]	4.5 ± 0.25 [a]	0.127	0.019
Calcium (mg/dL)	BT	12.04 ± 0.45	11.72 ± 0.70	11.84 ± 0.47	0.247	0.661
	AT	12.12 ± 0.43	12.2 ± 0.51	12.4 ± 0.38	0.199	0.605
Magnesium (mg/dL)	BT	2.22 ± 0.32	2.3 ± 0.29	2.28 ± 0.31	0.138	0.914
	AT	2.34 ± 0.27 [b]	2.42 ± 0.14 [ab]	2.68 ± 0.13[a]	0.086	0.040
Alanine transaminase (units/L)	BT	28 ± 1.87	27.6 ± 1.94	26.6 ± 2.79	1.003	0.609
	AT	31.6 ± 2.30	30.4 ± 2.96	29 ± 2.23	1.128	0.300
Aspartate transaminase (units/L)	BT	81.2 ± 3.84	79.6 ± 7.50	79.2 ± 6.87	2.807	0.868
	AT	127.6 ± 5.77 [a]	114 ± 10.93 [ab]	110.8 ± 11.69 [b]	4.393	0.043
Creatinine (mg/dL)	BT	1.34 ± 0.20	1.42 ± 0.28	1.32 ± 0.13	0.097	0.749
	AT	1.62 ± 0.22	1.58 ± 0.08	1.46 ± 0.18	0.078	0.355
Urea nitrogen (mg/dL)	BT	10.4 ± 2.30	11.2 ± 1.92	10.8 ± 2.58	1.023	0.859
	AT	13.6 ± 1.51	12.4 ± 2.07	11.8 ± 1.48	0.765	0.276

BT, before treatment; AT, after treatment.
or abbreviations, see Table III.

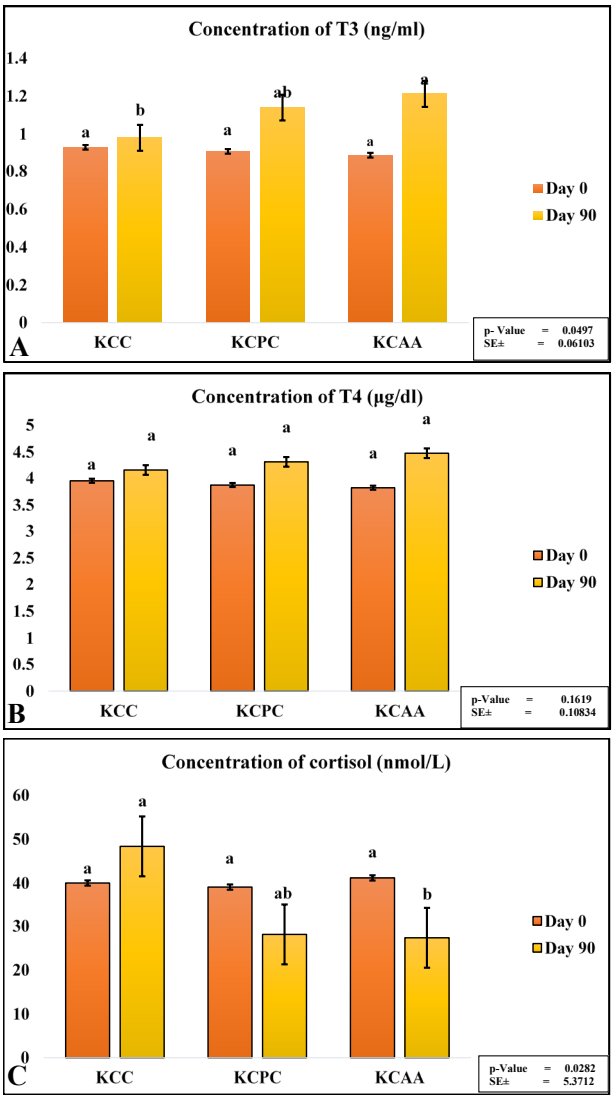


Fig. 2. Effects of ascorbic acid on concentration of T3, T4 and cortisol (nmol/L) hormone in KC, Kachhi control; KPC, Kachhi positive control; KAA, Kachhi ascorbic acid; Values marked with different superscript letters (a, b) indicates significant differences ($p<0.05$).

DISCUSSION

Maintaining a stable body temperature is an essential for homeothermic animals to sustain optimal physiological functions and productivity. Deviations from this range, especially in hot environments, can disrupt physiological responses and reduce performance (Hansen, 2014). Heat stress triggers physiological, biochemical, and behavioral adaptations to mitigate adverse effects (Silanikove, 2000). Under heat stress, animals activate thermoregulatory mechanisms to maintain thermal balance; however, extreme conditions can impair heat dissipation, negatively affecting well-being and productivity (Rivington *et al*., 2009).

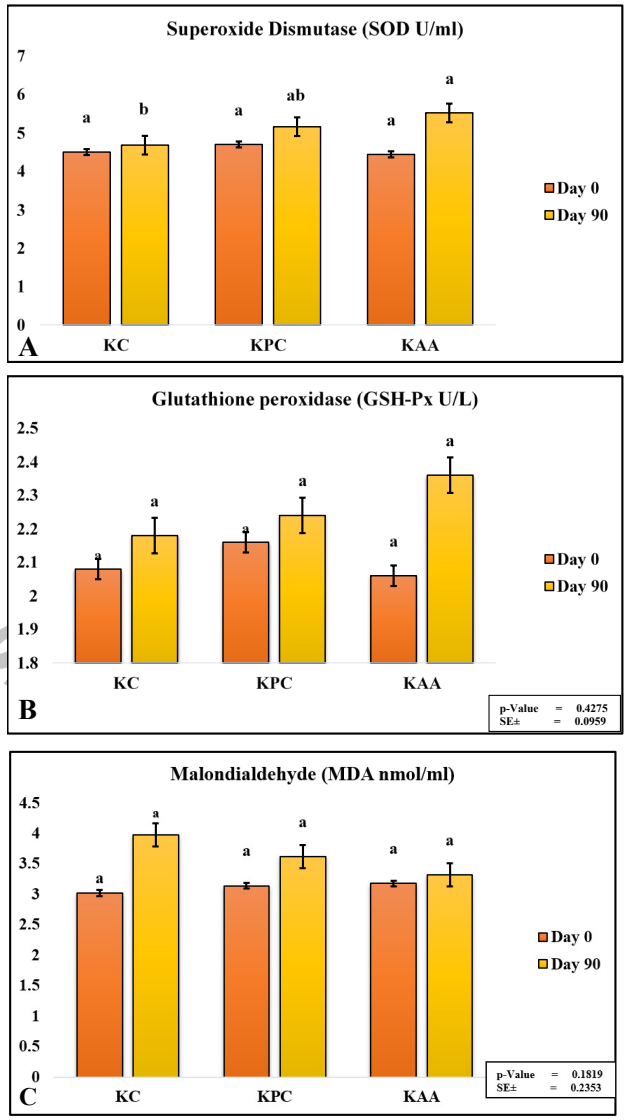


Fig. 3. Effects of ascorbic acid on concentration of SOD (A), GSH-Px (B), and Malondialdehyde (C) (MDA nmol/ml) as influenced by different management systems. KC, Kachhi control; KPC, Kachhi positive control; KAA, Kachhi ascorbic acid; Values marked with different superscript letters (a, b) indicates significant differences ($p<0.05$).

A critical adaptive response to heat stress involves HSPs, particularly HSP-70 and HSP-90, which play essential role in protein folding, apoptosis, immune regulation, and thermos-tolerance (Hassan *et al*., 2019). In this study, HSP-70 and HSP-90 mRNA expression

remained unchanged in KC lambs (fold change 1.00) but was significantly downregulated in KPC (0.51; 0.75) and KAA (0.36; 0.39) lambs. These findings align with studies by Dangi *et al*. (2014), Younis (2020) which reported elevated HSP-70 expression in heat-stressed sheep and goats. Similarly, Sharma *et al*. (2013) and Rout *et al*. (2016) linked higher THI values to increased HSP level, indicating a physiological adaptation to heat stress.

Heat stress induces physiological changes such as elevated rectal temperature, pulse rate, and respiration rate, which serve as indicators of adaptation to high environmental temperature (Marai *et al*., 2007). The heat stress response involves two primary mechanisms: heat dissipation (sweating, vaporization) and heat load regulation such as metabolic heat, radiation and convection (West, 2003). Disruptions in these mechanisms lead to increased respiration rate, pulse rate, and rectal temperature, which negatively impact animal performance and well-being (Ilori *et al*., 2011). In this study, KC lambs (exposed to 35–45°C) exhibited higher physiological stress markers, while KPC and KAA lambs showed reduced stress level. This aligns with findings by Sivakumar *et al*. (2010) and Abduallah and Zanouny (2014), who reported that AA supplementation significantly lowered stress indicators. However, studies by Alam *et al*. (2011) and Panda *et al*. (2016) reported non-significant changes in rectal temperature in goats but increased respiration and pulse rate, highlighting species-specific differences. Variability in responses, such as decreased heart rate (Al-Haidary, 2004) or non-significant changes (Aharoni *et al*., 2003), underscores the complexity of thermoregulatory mechanisms across species and conditions. Rashid *et al*. (2013) also found cyclic heat exposure significantly lowered heart rate, further illustrating the diverse nature of heat stress responses.

Hematological parameters are crucial indicators of physiological status and stress adaptation in animals. In this study, KAA lambs supplemented with AA showed significant improvements in RBCs, Hb, and PCV compared to KC and KPC groups, suggesting AA alleviates heat stress effects. The decline in these parameters in KC lambs may be attributed to oxidative damage to RBC membrane or reduced nutrient availability for Hb synthesis due to lower feed intake during heat stress (Srikandakumar *et al*., 2003). These findings are consistent with Abdul-Kareem (2011), who observed increased RBCs, Hb, and PCV with AA supplementation but reduced WBCs. Similarly, Wojtas *et al*. (2014) noted a decrease in WBCs under heat stress, and Mwafq *et al*. (2010), and Vijai *et al*. (2019) reported significant reductions in Hb and PCV during heat stress. Additionally, McManus *et al*., (2009) identified Hb and PCV as effective heat stress markers. However, other studies have reported contrasting findings. Sanusi *et al*. (2010) and Rashid *et al*. (2013) observed increased hematological parameters under heat stress, possibly due to breed and age differences. Similarly, Alam *et al*. (2011) and Attia and Noura (2016) reported significant increase in RBCs, WBCs, Hb, and PCV under heat stress, which contrasts with the current study. Conversely, Broucek *et al*. (2009) found no adverse effects on blood parameters in heat-stressed calves, reinforcing species-specific differences. Additionally, non- significant changes were observed in MCV, MCH or MCHC, aligning with Al-Haidary (2004) and Biobaku *et al*. (2016).

Elevated temperature impairs thyroid function by reducing metabolic rate and increasing H_2O_2, inhibiting hormone synthesis and T4-to-T3 conversion (Usha *et al*., 2002; Zengkui *et al*., 2019). Cortisol rises during heat stress, aiding acute responses (Barnes *et al*., 2004). In this study, AA-supplemented KAA lambs showed higher T3 and lower cortisol than KCC lambs, indicating reduced stress and improved thyroid function, consistent with findings in sheep and goats (Mwafq *et al*., 2010; Sivakumar *et al*., 2010; Omidi *et al*., 2014). However, Sejian *et al*. (2012) and Vijai *et al*. (2019) reported similar declines in T3 and T4 under heat stress, linked to HPA axis disruption. However, discrepancies in cortisol responses, such as Ashutosh and Kundu (2000) finding non-significant correlation in Indian sheep, may arise from study design or environmental variations.

Oxidative stress markers like SOD, GPx, and MDA reflect redox balance and cellular damage. Heat stress disrupts this balance, while antioxidants like AA boost defenses (Halliwell and Gutteridge, 2015). SOD neutralizes superoxide radicals, GPx reduces peroxides, and MDA indicates lipid peroxidation (Surai, 2002; Ayemele *et al*., 2021). In this study, the KAA group showed higher SOD, consistent with findings by Khan *et al*. (2020), and Abeyta *et al*. (2023). However, GPx level was numerically higher but non-significant, aligning with Chauhan *et al*. (2021) and Guo *et al*. (2021) MDA level was lower in KAA, though not significant, suggesting reduced lipid peroxidation, as noted by Ayemele *et al*. (2021) and Abeyta *et al*. (2023).

Serum biochemical parameters reflect animal health and metabolic status. In this study, glucose level were significantly higher in KPC, followed by KAA and KC lambs, aligning with findings by Cwynar *et al*. (2014) who noted reduced glucose under heat stress due to limited nutrient supply or increased energy demand. Total protein level was highest in KAA lambs, consistent with Helal *et al*. (2010) and Dangi *et al*. (2014) and who linked heat stress to reduced protein level, while Okoruwa (2014) reported increases due to dehydration. Potassium and magnesium level increased significantly in KAA lambs, aligning with

Chauhan and Agarwal (1995) and AbdAllah and Zanouny (2014), who found AA improved electrolyte balance. Sodium and calcium level were unaffected, though Al-Haidary (2004) noted decreases in heat-stressed goats due to sweating. Current study showed non-significant difference in ALT, creatinine, and urea nitrogen levels, though KC lambs showed higher values. AST level were significantly highest in KC, consistent with Sharma *et al*. (2011) and Rashid *et al*. (2013), who linked elevated AST to heat stress. However, Hamzaoui *et al*. (2013) reported non- significant changes in ALT or urea nitrogen.

However, some limitations must be considered. The sample size was relatively small, which may affect the robustness of statistical analyses. The study was conducted over a limited 90-days period, which might not reflect long-term physiological or productivity responses. Additionally, only one sheep breed (Kachhi) was used, limiting the generalizability of results to other genetic types or production systems. Further studies are needed to confirm these findings under varied environmental and genetic conditions.

CONCLUSION

Based on the above results it was concluded in the end that, physiological, hematological, hormonal and oxidative stress parameters were improved and down-regulation in *HSP-70* and *HSP-90* gene expression has been observed in Kachhi lambs fed with ascorbic acid in addition to green fodders and concentrate ration at animal shed. Hence, ascorbic acid can be used as anti-heat stress agents. Future studies should assess different doses of ascorbic acid, extend the study duration, and evaluate reproductive, immune, and productivity responses across various breeds and management systems to better address heat stress in sheep production.

DECLARATIONS

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IRB approval

The experimental work was approved by the Directorate of Advanced Studies, Sindh Agriculture University Tandojam, as part of a PhD research trail (Approval No: DAS/90, Dated: 13/01/2023).

Ethical approval

All animal care and slaughter procedures were approved by the Institutional Ethical Committee of Sindh Agriculture University, Tandojam, following ethical research guidelines.

Statement of conflict of interest

The authors have declared no conflict of interest.

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