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Impact of grazing duration on stress biomarkers and heat shock protein (HSP) expression in Kachhi sheep

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Abstract

This study examined the impact of varying grazing durations on stress biomarkers and heat shock protein (HSP) expression in Kachhi sheep under heat stress conditions. Fifteen lambs were randomly assigned into three groups (n=5): morning grazing (KMG; 7:00 AM–11:00 AM), hot hours grazing (KHG; 11:00 AM–3:00 PM), and evening grazing (KEG; 3:00 PM–7:00 PM), with all lambs receiving a basal diet of green fodder and concentrate in addition to grazing. The 90-day trial included fortnightly recording of physiological parameters (rectal temperature, pulse rate, and respiration rate), as well as blood sampling on days 0 and 90 for hematological, hormonal, biochemical, oxidative stress, and gene expression analysis. Rectal temperature, pulse rate, and respiration rate were recorded using standard methods; rectal temperature was significantly higher ($p<0.05$) in KHG lambs, while pulse and respiration rates did not differ significantly ($p>0.05$). Hematological parameters (RBC, Hb, PCV, WBC, MCV, MCH, MCHC), analyzed using an automatic Nihon Kohden analyzer, showed non-significant differences ($p>0.05$) among the groups. Hormonal level (T3, T4, cortisol), measured by Electrochemiluminescence Immunoassay (ECLIA) on a Cobas-e analyzer, revealed higher T4 in KMG and KEG lambs. In contrast, T3 and cortisol levels were not significantly different ($p>0.05$) across groups. Oxidative stress parameters (SOD, GSH-Px, MDA), assessed using assay kits, also showed non-significant variation ($p>0.05$). Serum biochemical parameters, analyzed using a Roche Hitachi C311 analyzer, indicated significant differences ($p<0.05$) in glucose, total protein, sodium, and potassium. At the same time calcium, magnesium, AST, ALT, creatinine, and urea nitrogen levels were not significantly affected ($p>0.05$). Gene expression of HSP-70 and HSP-90, determined by quantitative real-time PCR (qRT-PCR), showed significant upregulation in KHG lambs and downregulation in KMG and KEG lambs. These findings suggest that morning grazing mitigates heat stress in Kachhi lambs by improving physiological responses, stabilizing hormonal levels, and reducing cellular stress, thereby enhancing overall health and performance.

Keywords: Grazing management, Heat stress, HSP-70 and 90, Kachhi sheep, Stress biomarkers

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Introduction

Heat stress poses a significant challenge to livestock production, particularly in arid and semi-arid regions with high ambient temperatures (El-Tarabany et al., 2017). It occurs when an animal's heat load surpasses its capacity to dissipate excess heat, leading to physiological and metabolic disruptions that impair growth, reproduction, and overall productivity. These disruptions result in reduced feed intake, lower milk yield, impaired fertility, and increased disease susceptibility. Endocrine imbalances particularly altered cortisol and thyroid hormone levels further contribute to reproductive and performance losses, making heat stress a significant cause of economic loss in the livestock sector (Collier et al., 2017). Sheep, like other mammals, rely on behavioral, physiological, and biochemical mechanisms to maintain thermal homeostasis. However, prolonged exposure to high temperatures can overwhelm these adaptive responses (Silanikove 2000). The thermo-neutral zone for sheep typically ranges between 12 °C and 32 °C. Deviations from this range, especially when combined with a high temperature-humidity index (THI), severely compromise their ability to cope with heat stress (Shelton 2000).

At the molecular levels, heat shock proteins (HSPs), particularly HSP-70 and HSP-90, play a crucial role in cellular protection and thermo-tolerance. HSP-70 facilitates protein folding, prevents denaturation and enhances cell survival under stress, while HSP-90 helps prevent irreversible protein aggregation (Chen et al., 2006; Sharma et al., 2013). Elevated extracellular level of HSP-70 have been observed in thermally stressed animals, underscoring its importance as a biomarker of cellular adaptation (Min et al., 2015). Physiological indicators such as increased body temperature, respiration rate, and altered hematological and

biochemical profiles are commonly used to assess heat stress in sheep (Srikandakumar et al., 2003; Marai et al., 2007). Heat stress can reduce red blood cell (RBC), hemoglobin (Hb) concentration and packed cell volume (PCV), likely due to oxidative damage, increased oxygen demand and reduced feed intake (Temizel et al., 2009; Sivakumar et al., 2010). Additionally, it disrupts endocrine function by suppressing thyroid hormone secretion (Nazifi et al., 2003; Rasooli et al., 2004) and elevating cortisol levels, which facilitate stress adaptation (Indus & Pareek, 2015).

Oxidative stress is another critical consequence of heat exposure, as it increases reactive oxygen species (ROS) production, which can damage cellular structures if not neutralized. Antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) play a vital role in mitigating oxidative damage (Halliwell & Gutteridge, 2015; Ayemele et al., 2021). Furthermore, heat stress disrupts electrolyte balance, induces hyperglycemia via cortisol-mediated gluconeogenesis, and impairs liver and kidney function, as evidenced by altered enzyme activities and elevated blood urea nitrogen levels (West, 1999; Bernabucci et al., 2002).

Grazing management is an important strategy to mitigate heat stress in livestock. The timing of grazing whether during cooler morning hours, peak heat periods, or late afternoon affects exposure to solar radiation and ambient temperature, thereby influencing stress responses. Studies suggest that grazing during cooler periods reduces heat load, lowers rectal temperature, improves feed intake, and enhances animal welfare (Tucker et al., 2007). Adjusting grazing schedules based on environmental conditions is a practical and cost-effective approach to minimizing heat stress in small ruminants (da Silva et al., 2016; Sejian et al., 2018). In arid and semi-arid regions, where heat stress is persistent, strategic grazing during thermally favorable periods may significantly improve livestock performance and physiological resilience (Rojas-Downing et al., 2017). Despite the importance of grazing management in reducing heat stress, there is limited research specifically addressing how different grazing times influence stress biomarkers and physiological responses in indigenous sheep breeds such as the Kachhi. Therefore, this study was conducted to fill this knowledge gap and provide scientific justification for optimizing grazing schedules under hot climatic conditions. The findings are expected to support the development of low-cost, effective management strategies for improving animal health, welfare, and productivity in heat-stressed environments. Given these considerations, this study aimed to evaluate the effects of different grazing durations on the expression of *HSP-70* and *HSP-90* genes, as well as changes in physiological, hematological, hormonal, oxidative stress, and biochemical parameters in Kachhi sheep exposed to hot climatic conditions.

Materials and Methods

Ethical approval

All animal care and procedures were approved by the Institutional Ethical Committee of Sindh Agriculture University, Tandojam in accordance with ethical research guidelines (Ethical Approval No. DAS/90/2023, dated January 13, 2023).

Experimental animals and their management

The study was conducted from May to July at Sindh Agriculture University Tandojam, Department of Livestock Management and 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 the surrounding districts of Sindh. They were housed in well-ventilated sheds with free access to fresh water. Along with grazing all lambs received a basal diet of 70% roughage and 30% concentrates, containing approximately 60-70% moisture, 15-20% crude protein, 25-35% crude fiber, and 3-6% ether extract.

Experimental measurements

The experimental trial was conducted for 90 days, including a 15-days adaptation period, with lambs randomly divided into three groups (n=5) based on their body weight. The morning hours grazing group (KMG) was allowed to graze in the livestock field from 7:00 AM to 11:00 AM, during which the ambient temperature ranged between **(**30 to 34 °C ± 2.0)**.** The hot hours grazing group (KHG) had access to grazing during the hottest part of the day, from 11:00 AM to 3:00 PM, when the ambient temperature ranged between **(**42 to 45 °C ± 2.5)**.** The evening hours grazing group (KEG) was allowed to graze from 3:00 PM to 7:00 PM, with ambient temperatures ranging from **(**36 to 39 °C ± 1.5)**.** The environmental conditions were monitored with the help of Automatic Temperature Clock Humidity (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) (Table 1).

Table 1 Monthly average ambient temperature, relative humidity, and thermal humidity index (THI) during the experimental period

Parameters	May	June	July
Average Ambient temperature (°C)	42.96 ± 0.34	41.43 ± 0.29	39.90 ± 0.31
Average Relative Humidity (%)	37 ± 1.12	38 ± 1.05	40 ± 1.20
Thermal Humidity Index (THI)	91.34 ± 0.52	89.82 ± 0.48	88.52 ± 0.50

RH = Relative humidity, THI = Temperature humidity index

Blood collection and serum separation

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.

Expression pattern of HSP-70 and 90 proteins

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 2). GAPDH was used as the reference gene for normalization, and gene expression was analyzed using the 2-ΔΔCT method (Livak & Schmittgen, 2001) on an AB Applied Biosystems 7300 system. All reactions were performed twice 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 are provided in (Table 3). PCR amplification was verified using 1% agarose gel electrophoresis and post-PCR melt curve analysis.

Table 2 Primers used for HSP-70 and HSP-90 expression. GAPDH used as reference gene to normalize the gene expression of target genes

Desired gene	Sequence (5′ →3′)	Primers	Primer Length	Accession number
HSP-70	GACAAGTCGGAGAACGTGCA	Forward	20	JN604434
HSP-70	CGTACACCTGGATCAGCAC	Reverse	19	
HSP-90	ATTGACATCATCCCGAATC	Forward	19	EF091713
HSP-90	ACACCAAACTGCCCAATCAT	Reverse	20	
GAPDH	GCAAGTTCCACGGCACAGTC	Forward	20	AF030943
GAPDH	CCCACTTGATGTTGGCAGGA	Reverse	20	

HSP70 - Heat Shock Protein 70; HSP90 - Heat Shock Protein 90; GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

Table 3 PCR conditions

Cycle steps	Temperature °C	Time	Number of cycles
Reverse transcription	50°C	15 minutes	
Initial denaturation	95°C	10 minutes	
Denaturation	95°C	10 seconds	45 Cycles
Annealing	53°C	30 seconds	
Extension	72°C	30 seconds	
Cooling	60°C	1 minute	

Physiological parameters

The physiological parameters were recorded as per method described by Reece et al. (2015). Rectal temperature of lambs was taken in Fahrenheit (°F) by inserting the thermometer 6–7 cm through the rectum. The respiration rate was recorded as breaths/minute by counting movements of the flank region with the help of a stopwatch. However, the pulse rate of lambs was noted as beats/minute by palpating the femoral artery.

Hematological parameters

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 parameters

Hormonal concentrations of triiodothyronine (T3), thyroxine (T4), and cortisol were measured via Electrochemiluminescence Immunoassay (ECLIA) using a Cobas e immunoassay analyzer as described by (Sanchez-Carbayo et al., 1994).

Oxidative stress parameters

Oxidative stress markers, including superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA), were analyzed using assay kits from Nanjing Jincheng Bioengineering Institute (China).

Serum biochemical parameters

Serum biochemical parameters, including 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 (Al-Haidary et al., 2012). All procedures strictly followed the manufacturers' protocols to ensure accuracy and reliability

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 (Glyceraldehyde 3-phosphate dehydrogenase), were analyzed using one-way ANOVA. The means for each group were compared using Tukey's HSD post-hoc test.

Results and Discussion

Gene expression analysis of HSP-70 and HSP-90 genes

Quantitative real-time PCR (qRT-PCR) analysis showed unchanged HSP-70 and HSP-90 expression in the KMG group (fold change: 1.00) and down-regulation in KEG-evening hours grazing (Fold change 1.07; 1.15) and up-regulation in KHG-hot hours grazing lambs (Fold change 1.62; 1.83) were observed. On a comparative basis, the blood HSP-70 and HSP-90 expression were significantly ($p<0.05$) higher in KHG lambs compared to both KMG and KEG lambs (Table 4). Melt curve analysis (Applied Biosystems 7300) indicated higher expression in KHG, with lower curves in KMG and KEG (Fig. 1). Agarose gel electrophoresis (Fig. 2) confirmed this pattern, showing stronger HSP-70 and HSP-90 bands in KHG, with reduced expression in KMG and KEG lambs. These results are consistent with Guzmán et al. (2023), who observed lower HSP-70 gene expression in the morning and higher expression in the afternoon in Simmental cattle. Similarly, the findings are in line with Deb et al. (2014) who found elevated HSP-90 expression in Sahiwal cattle during the summer, with a marked increase at 45°C compared to 39 °C. Furthermore, the results align with Kishore et al. (2014) they found that HSP expression levels increased in cows after two hours of sun exposure, while Maibam et al. (2017) noted constitutive upregulation of HSP-70 expression in Tharparkar cattle during warmer hours of the day. In support of these findings, Min et al. (2015) documented significant increases in serum concentrations of Heat Shock Factor and HSPs 27, 70, and 90 in heat-stressed dairy cows. Further corroborating these trends, Chauhan et al. (2014) reported a 3.5-fold increase in HSP-70 mRNA expression in skeletal muscle under heat stress, though HSP-90 expression was not significantly affected. Baek et al. (2019) also confirmed that heat stress induces HSP expression and secretion into extracellular spaces and blood in Hanwoo cattle. In goats, Dangi et al. (2014; 2012) reported significant ($p<0.05$) seasonal variation in HSP-90 expression, particularly in the summer, while HSP-70 expression increased significantly ($p<0.05$) only in goats from tropical regions. Moreover, Banerjee et al. (2014) also noted seasonal fluctuations in HSP-70 gene expression across different goat breeds adapted to diverse agro-climatic conditions. However, the current findings contrast with those of Aritonang et al. (2017), who found that HSP-90 expression was not significantly affected by differences in environmental conditions during the early and afternoon hours. Such discrepancies may arise due to species differences, variation in experimental conditions, or differences in study design and duration.

Table 4 Gene expression analysis of HSP-70 and HSP-90 genes as influenced by grazing management

Parameter	KMG	KHG	KEG	p-value
HSP-70 (average Ct)	38.50 ± 0.30	39.80 ± 0.40	38.90 ± 0.25	-
GAPDH (average Ct)	38.41 ± 0.35	38.46 ± 0.31	38.07 ± 0.47	-
ΔCt°	0.09 ± 0.06	1.34 ± 0.15	0.02 ± 0.05	-
$\Delta\Delta Ct^{\#}$	0.00 ± 0.002	1.25 ± 0.10	-0.07± 0.05	-
Fold change	**1.00** [a]	**1.62** [c]	**1.07** [b]	<0.0001
HSP-90 (average Ct)	38.50 ± 0.35	39.61 ± 0.49	38.20 ± 0.30	-
GAPDH (average Ct)	38.10 ± 0.40	38.47 ± 0.35	38.10 ± 0.27	-
ΔCt°	0.04 ± 0.10	1.14 ± 0.15	0.1 ± 0.10	-
$\Delta\Delta Ct^{\#}$	0.0 ± 0.001	1.1 ± 0.05	0.06 ± 0.02	-
Fold change	**1.00** [a]	**1.83** [c]	**1.15** [b]	<0.0001

KMG = Kachhi morning grazing; KHG = Kachhi hot hours grazing; KEG = Kachhi evening grazing; Values denoted by a different superscript letter (a,b) differ significantly ($p<0.05$).

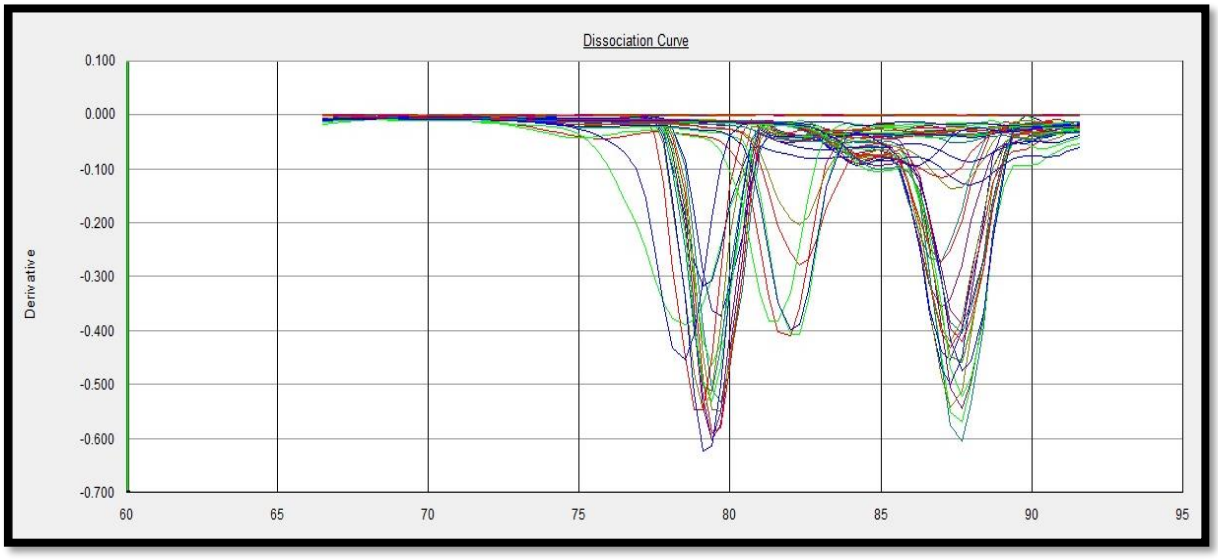

Fig. 1 Melt curve analysis of qRT-PCR amplification products for target and reference genes

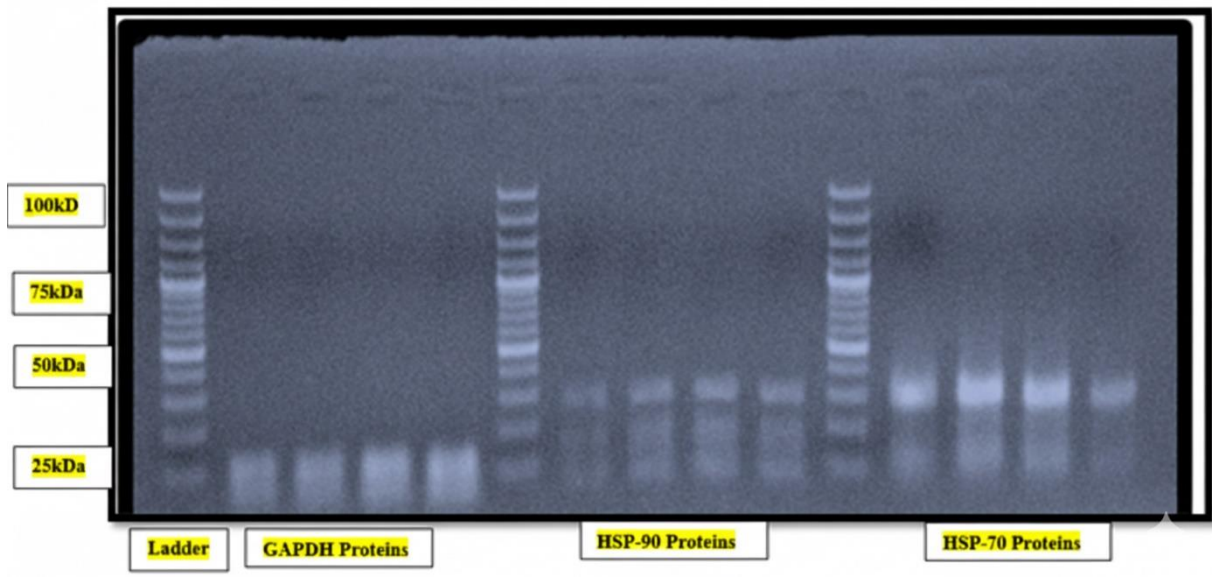

Fig. 2 Agarose gel electrophoresis of qRT-PCR products

Physiological responses (Rectal temperature, pulse rate and respiratory rate)

The results of the physiological responses are shown in (Table 5). It was observed that allowing KHG lambs to graze during the hottest parts of the day resulted in a significant ($p<0.05$) increase in rectal temperature and reduced in KMG and KEG lambs grazed in the morning and evening hours of the day. However, the findings showed that pulse rate and respiration rate did not differ significantly. These findings align with previous reports by Karthik et al. (2021) and Sejian et al. (2012), who found increased physiological parameters particularly rectal temperature, pulse rate, and respiration rate in sheep grazing during hotter periods or under semi-arid conditions. Similarly, Kochewad et al. (2017) and Hyder et al. (2017) attributed elevated pulse and respiration rates to longer grazing distances and increased metabolic activity under semi-intensive and extensive systems. These results align with Kalyan et al. (2023) who found significantly ($p<0.05$) higher physiological parameters in lambs exposed to heat stress compared to controls. Contrastingly, Maulana et al. (2022) observed lower pulse and respiration rates in sheep with outdoor access, suggesting enhanced comfort in extensive systems. The physiological mechanisms underpinning these responses such as increased oxygen intake and blood flow to the skin aid in heat dissipation (Habibu et al., 2017; Adedeji, 2012). In line with the current results, previous studies by Sejian et al. (2013) have also shown that pulse rate tends to rise during the midday period, reflecting a clear diurnal pattern under heat stress conditions.

Table 5 Physiological responses as influenced by grazing management

Parameter	KMG	KHG	KEG	SE	p-Value
Rectal temperature (°F)	102.4 ± 0.54^{b}	103.6 ± 0.54^{a}	102.8 ± 0.83^{ab}	0.294	0.038
Pulse rate (beat/ min)	71.8 ± 4.60	77.2 ± 3.11	75.6 ± 2.50	1.574	0.082
Respiration rate (breath/min)	24.4 ± 1.51	27.6 ± 2.88	26.2 ± 1.92	0.976	0.107

KMG = Kachhi morning grazing; KHG = Kachhi hot hours grazing; KEG = Kachhi evening grazing; SE = Standard error; Values denoted by a different superscript letter (a, b) differ significantly.

Determination of Hematological parameters

This study evaluated the impact of three distinct grazing strategies morning hours grazing (KMG), hot hours grazing (KHG), and evening hours grazing (KEG) on the hematological profile of Kachhi lambs. Parameters assessed included red blood cell count, hemoglobin (Hb) concentration, packed cell volume (PCV), white blood cell count (WBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) (Table 6). Statistical analysis revealed no significant differences among the groups, indicating that grazing time had a minimal impact on hematological values. This stability suggests that none of the grazing schedules induced physiological stress, and all parameters remained within normal physiological ranges. RBC, Hb, and PCV values which reflect oxygen transport and metabolic status remained stable, as did MCV, MCH, and MCHC, suggesting unaffected erythrocyte morphology and hemoglobin content. These findings are consistent with El Huda (2018) and Adenkola and Ayo (2010) who reported that moderate environmental or management variations have little effect on hematological indices in small ruminants. Though heat stress during hot-hour grazing could theoretically alter these parameters (Marai et al., 2007), the lack of significant changes in the present study highlights the adaptive capacity of Kachhi lambs, a breed well-suited to arid and semi-arid climates. Furthermore, the provision of supplemental green fodder and concentrate likely buffered against potential nutritional stress, supporting hematological stability. The present findings are supported by Das et al. (2024), who noted that proper

nutritional management plays a vital role in preserving physiological homeostasis in animals facing environmental challenges. Contrasting findings reported by Addass et al. (2012); Maulana et al. (2022) regarding hematological values in intensively and extensively managed animals may be due to differences in breed adaptability, climatic conditions, and experimental setups. Variations in genetic traits, temperature and humidity levels, as well as management practices such as feeding, health care, and sampling methods, likely influenced the observed results. Similarly, Karthik et al. (2021) reported seasonal variations in hematological traits, and studies by Banerjee et al. (2014); Singh et al. (2016); Habibu et al. (2017) and observed lower RBC, Hb, and PCV in grazing sheep compared to those under other systems. These discrepancies highlight the influence of breed-specific resilience, environmental conditions, and dietary factors. Overall, the present findings underscore the adaptability of Kachhi lambs to varied grazing schedules and emphasize the importance of nutritional support in maintaining physiological health under different management practices.

Table 6 Hematological parameters as influenced by grazing management

Parameters		Groups				
		KMG	KHG	KEG	SE	p-Value
Red blood cells (10^6/µL)	BT	8.26 ± 0.87	8.54 ± 0.87	8.30 ± 0.73	0.372	0.849
	AT	9.82 ± 0.92	8.94 ± 0.65	9.36 ± 0.76	0.353	0.251
Hemoglobin (g/dl)	BT	8.64 ± 0.87	8.74 ± 0.87	8.8 ± 0.54	0.349	0.948
	AT	9.66 ± 0.87	9.28 ± 0.55	9.8 ± 0.54	0.302	0.475
Packed cell volume (%)	BT	25.6 ± 2.40	26.4 ±3.20	26.2 ± 3.11	1.311	0.904
	AT	29.2 ± 1.92	27.8± 1.92	27.6 ± 1.94	0.864	0.390
White blood cells (10^3/ µL)	BT	10.02 ± 0.97	10.56 ± 0.79	10.38 ± 1.02	0.419	0.660
	AT	10.52 ± 0.54	11.18 ± 0.43	10.66 ± 0.54	0.226	0.137
Mean corpuscular volume (fL)	BT	31.35 ± 5.31	30.98 ± 3.09	31.64 ± 3.52	1.831	0.968
	AT	30.0 ± 4.06	31.23 ± 1.47	29.67 ± 3.42	1.613	0.777
Mean corpuscular hemoglobin (pg)	BT	10.56 ± 1.65	10.29 ± 1.23	10.67 ± 1.25	0.623	0.906
	AT	9.89 ± 1.22	10.43 ± 0.99	10.55 ± 1.28	0.524	0.654
Mean corpuscular hemoglobin Concentration (g/dL)	BT	33.83 ± 3.09	33.54 ± 5.51	33.84 ± 3.31	1.843	0.991
	AT	33.17 ± 3.55	33.54 ± 3.55	35.55 ± 1.21	1.336	0.425

KMG = Kachhi morning grazing; KHG = Kachhi hot hours grazing; KEG = Kachhi evening grazing; SE = Standard error; Values denoted by a different superscript letter (a, b) differ significantly ($p<0.05$); BE = Before treatment; AT = After treatment

Hormonal parameters

This study investigated the effects of different grazing times on the hormonal profile of Kachhi lambs, specifically focusing on Triiodothyronine (T3), Thyroxine (T4), and cortisol. The results revealed higher T4 in KMG and KEG lambs, while T3 and cortisol level were not significantly different across groups (Fig. 3, 4 and 5). These results are in agreement with Aleena et al. (2016), who reported reduced thyroid hormone synthesis during heat stress due to suppression of the hypothalamic-pituitary-thyroid (HPT) axis. Similarly, Indu et al. (2014); Ribeiro et al. (2016); Habibu et al. (2017) observed seasonal reductions in thyroid hormone levels in sheep managed under semi-intensive and extensive systems during the summer. Furthermore Karthik et al. (2021) and Khan et al. (2020) also noted higher T3 and T4 concentrations in sheep managed semi-intensively compared to extensively managed animals, consistent with the current findings. Cortisol, a glucocorticoid hormone secreted in response to stress via activation of the hypothalamic-pituitary-adrenal (HPA) axis, showed no significant differences among the groups. Nonetheless, numerically higher cortisol levels were observed in KHG lambs. This is supported by Krishnan et al. (2023), who reported that under heat stress, stimulation of skin thermoreceptors triggers the release of cortisol through activation of the HPA axis, which assists in cooling the body by promoting blood vessel expansion. Furthermore, Narayan and Parisella (2017) also emphasized cortisol as a key marker of stress response. Similarly, Liu et al. (2022) further reported significantly higher cortisol levels in sheep exposed to direct sunlight compared to those provided with shade. These findings highlight the importance of adaptive grazing strategies in mitigating hormonal disruptions caused by thermal stress. Grazing during cooler periods appears to support thyroid hormone stability and limit cortisol elevation, thereby enhancing metabolic efficiency and promoting animal welfare in hot climates.

Oxidative stress parameters

This study evaluated oxidative stress parameters, including superoxide dismutase (SOD), glutathione peroxidase (GPx), and malondialdehyde (MDA) in Kachhi lambs subjected to different grazing management strategies. The results indicated a non-significant difference in the concentrations of superoxide dismutase, glutathione peroxidase and malondialdehyde among the groups (Fig. 6, 7 and 8). These biomarkers serve as important indicators of the physiological response to environmental stressors, particularly heat. SOD is a critical antioxidant enzyme that catalyzes the dismutation of superoxide radicals into oxygen and hydrogen peroxide, playing a vital role in cellular defense against oxidative stress. Although the differences in SOD activity among the grazing groups were not statistically significant, numerically higher levels were observed in the KHG group compared to the KMG and KEG groups. This trend suggests that lambs grazing during peak heat periods may experience increased oxidative stress, thereby stimulating a greater enzymatic antioxidant response. These findings align with those of Saeed (2023), who reported elevated SOD activity in ruminants exposed to higher ambient temperatures. Similarly, Sharma et al. (2011) and Alberghina et al. (2024) emphasized that

enhanced SOD activity serves as an adaptive mechanism to counteract the accumulation of reactive oxygen species (ROS) in heat-stressed animals. GPx, another essential component of the antioxidant defense system, functions by reducing hydrogen peroxide and lipid peroxides, thereby protecting cells from oxidative damage. In the present study, GPx levels did not differ significantly across the grazing groups. However, numerically higher GPx activity in the KMG and KEG groups suggests a more favorable oxidative balance compared to KHG lambs. These results are consistent with the findings of Mirzad et al. (2018), who observed that ruminants grazing during cooler periods maintained more stable GPx activity. Likewise, Gallelli et al. (2018) found that providing animals with better thermal conditions helped boost antioxidant defenses like GPx, suggesting a reduction in heat-induced oxidative stress. Malondialdehyde (MDA) is not expressed as a protein but is a lipid peroxidation byproduct formed during oxidative damage to cell membranes, often serving as a biomarker for the extent of oxidative stress. MDA, a well-established marker of lipid peroxidation and oxidative membrane damage, also showed non-significant differences among the groups. Nevertheless, numerically higher MDA concentrations in the KHG lambs suggest increased oxidative damage due to prolonged heat exposure. This observation is in agreement with Jack et al. (2016), who reported elevated MDA levels in sheep exposed to high ambient temperatures. Furthermore, Guo et al. (2021) also found increased MDA concentrations in heat-stressed goats, attributing it to heightened ROS production and metabolic activity. Similarly, Chauhan et al. (2021) demonstrated that oxidative damage, as indicated by elevated MDA levels, was more pronounced in animals exposed to direct solar radiation compared to those managed under shaded or cooler conditions.

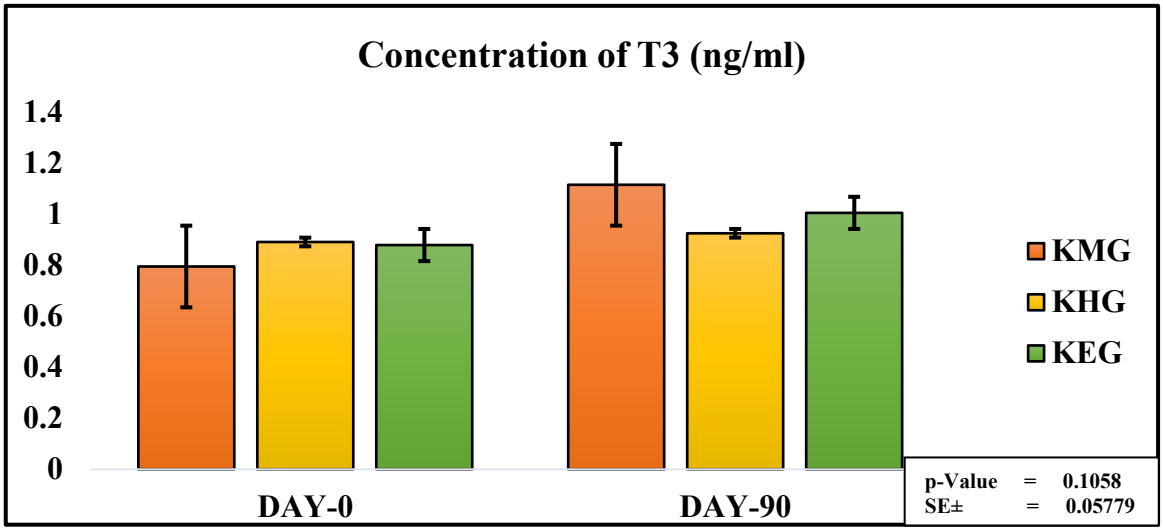

Fig. 3 Concentration of T3 (ng/ml) hormone. Graphical presentation of the concentration of the T3 hormone. The bars of the graphs represent the following groups: KMG – Kachhi morning grazing; KHG – Kachhi hot hours grazing; KEG – Kachhi evening grazing; Values denoted by a different superscript letter (a,b) differ significantly ($p<0.05$).

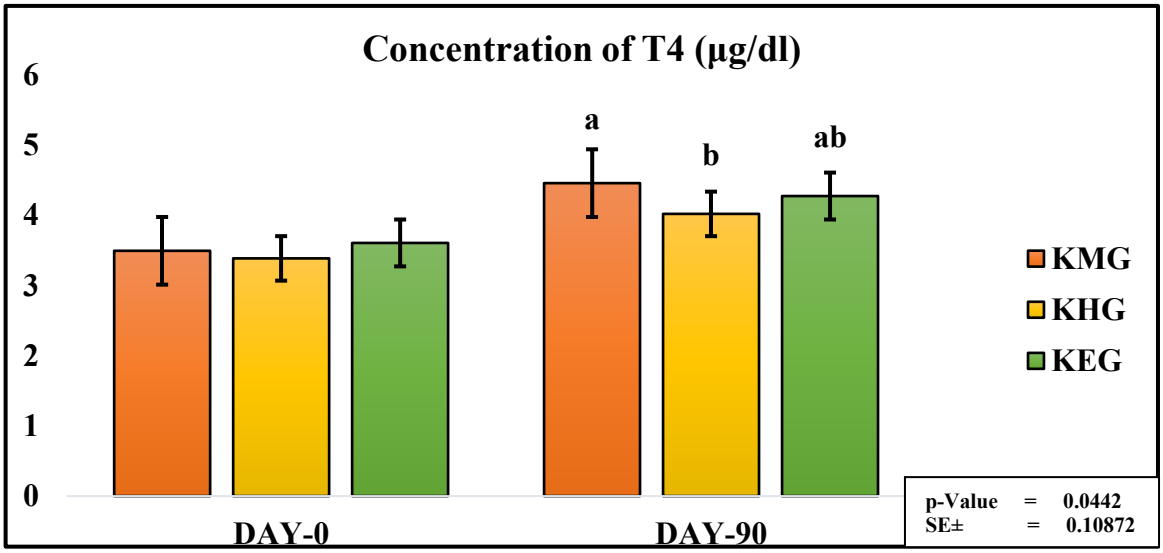

Fig. 4 Concentration of T4 (µg/Dl) hormone. Graphical presentation of the concentration of the T4 hormone. The bars of the graphs represent the following groups: KMG – Kachhi morning grazing; KHG – Kachhi hot hours grazing; KEG – Kachhi evening grazing; Values denoted by a different superscript letter (a, b) differ significantly ($p<0.05$).

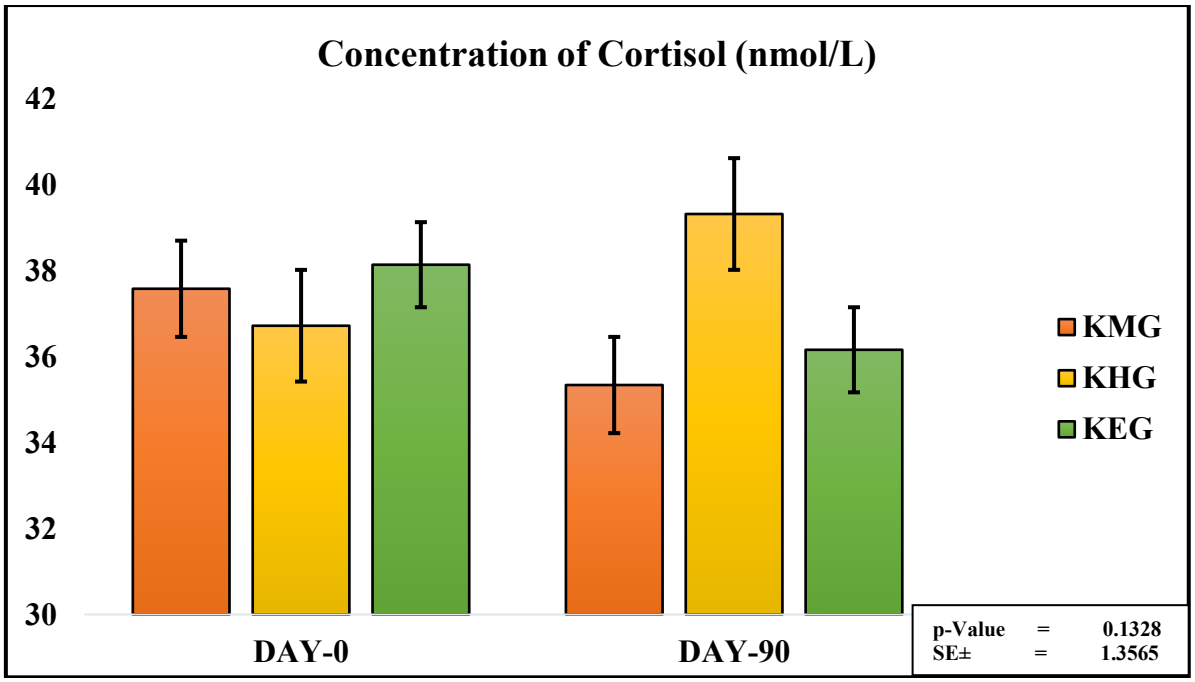

Fig. 5 Concentration of cortisol (nmol/L) hormone**.** Graphical presentation of the concentration of the cortisol hormone. The bars of the graphs represent the following groups: KMG – Kachhi morning grazing; KHG – Kachhi hot hours grazing; KEG – Kachhi evening grazing; Values denoted by a different superscript letter (a, b) differ significantly ($p<0.05$).

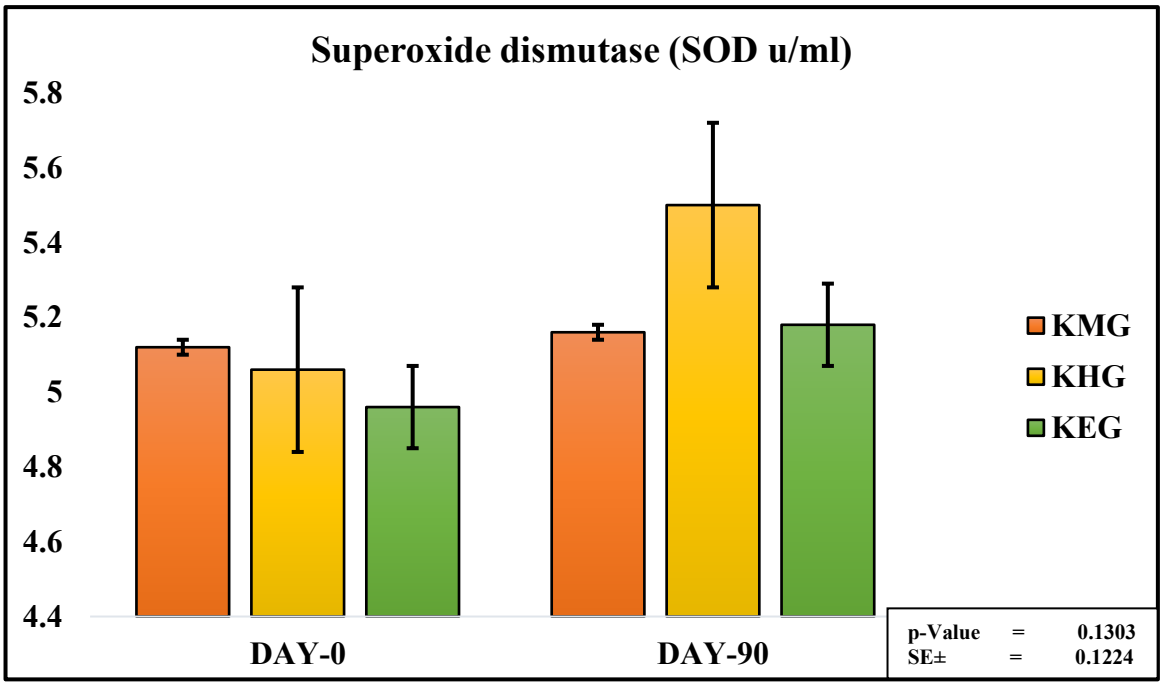

Fig. 6 Concentration of superoxide dismutase (SOD U/ml). Graphical presentation of the concentration of superoxide dismutase. The bars of the graphs represent the following groups: KMG – Kachhi morning grazing; KHG – Kachhi hot hours grazing; KEG – Kachhi evening grazing; Values denoted by a different superscript letter (a, b) differ significantly ($p<0.05$).

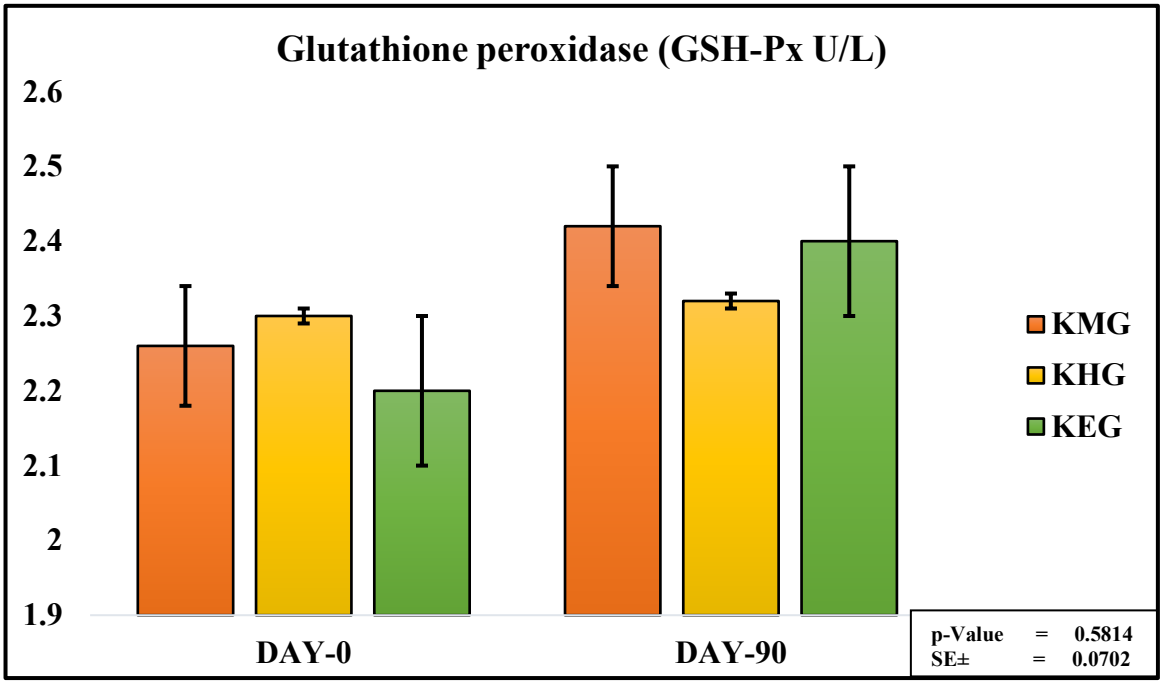

Fig. 7 Concentration of Glutathione peroxidase (GSH-Px U/L). Graphical presentation of the concentration of glutathione peroxidase. The bars of the graphs represent the following groups: KMG – Kachhi morning grazing; KHG – Kachhi hot hours grazing; KEG – Kachhi evening grazing; Values denoted by a different superscript letter (a, b) differ significantly ($p<0.05$).

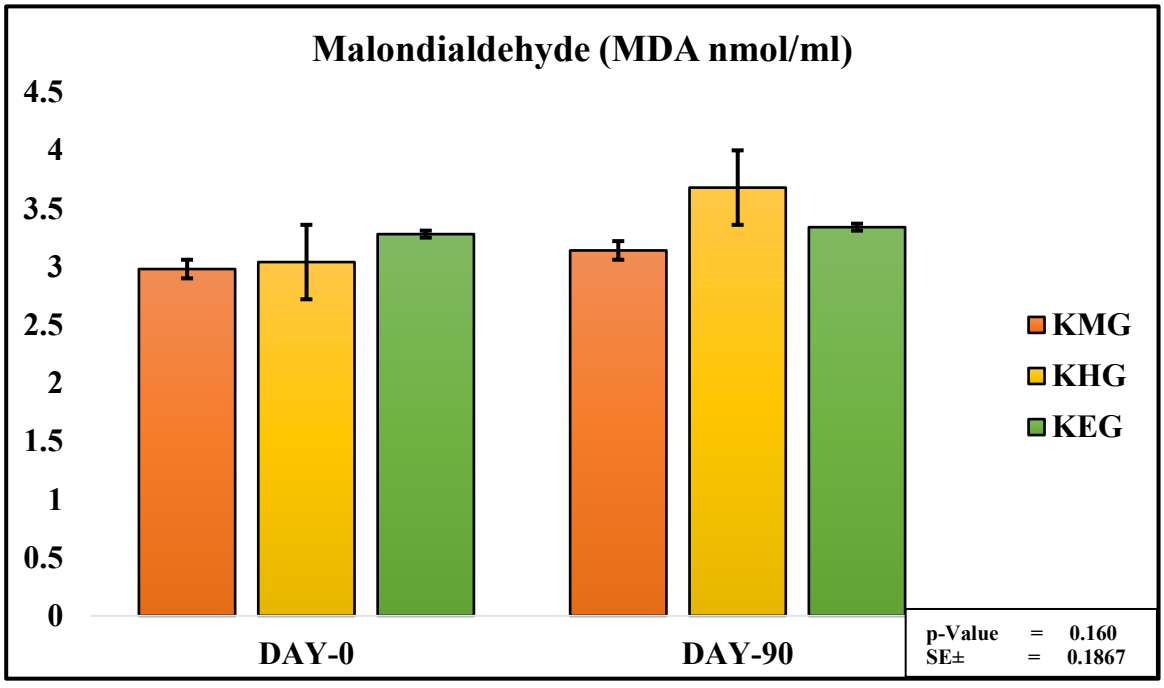

Fig. 8 Concentration of Malondialdehyde (MDA nmol/ml). Graphical presentation of the concentration of Malondialdehyde. The bars of the graphs represent the following groups: KMG – Kachhi morning grazing; KHG – Kachhi hot hours grazing; KEG – Kachhi evening grazing; Values denoted by a different superscript letter (a, b) differ significantly ($p<0.05$).

Serum biochemical parameters

The biochemical responses of Kachhi lambs to different grazing management systems morning (KMG), hot hours (KHG), and evening (KEG) revealed significant physiological variations among the groups. These results are presented in (Table 7). Lambs grazing during hot hours exhibited a significant increase in blood glucose levels. This elevation is likely a result of heat-induced stress, which triggers cortisol secretion and stimulates gluconeogenesis (Sejian et al., 2013). In contrast, lambs grazing during the cooler morning and evening hours showed lower glucose levels, indicative of reduced thermal stress and improved metabolic regulation. Similar findings have been reported by Mahjoubi et al. (2014) and Rashid et al. (2013) who observed increased glucose concentrations in heat-stressed

ruminants due to stress-mediated gluconeogenesis. These results support the view that grazing during cooler periods promotes metabolic stability by minimizing stress-induced glucose fluctuations (Sejian et al., 2018; Adenkola & Ayo, 2010). However, these findings contrast with those of Karthik et al. (2021), who reported lower serum glucose in sheep grazing for extended periods under hot conditions in extensive systems. Reduced glucose during summer grazing has also been attributed to decreased nutrient intake and thermal discomfort (Ramana et al., 2013). Such discrepancies may stem from differences in environmental conditions, grazing duration, dietary supplementation, and breed-specific adaptability, all of which influence energy metabolism and stress responses. In the current study, total protein levels were significantly ($p<0.05$) higher in lambs grazing during the evening hours. This increase may be attributed to enhanced nutrient digestion and absorption in cooler temperature, which favor protein metabolism. Elevated serum total protein levels could also result from more efficient conversion of non-protein nitrogen into amino acids and microbial protein. This may be supported by a higher population of rumen microbes and protozoa, which utilize available non-protein nitrogen and dietary urea (Jiao et al., 2024). Additionally, increased physical activity associated with grazing could enhance protein synthesis and metabolism (Ke et al., 2023). Conversely, lambs grazing during hot hours exhibited lower protein levels, likely due to reduced feed intake and thermal discomfort. These findings are consistent with Al-Dawood (2017), who highlighted that heat stress impairs protein metabolism in small ruminants, reinforcing the benefits of grazing during cooler periods. Sodium and potassium levels were significantly ($p<0.05$) elevated in lambs grazing during hot hours. These findings are in agreement with Sejian et al. (2010), who attributed increased sodium and potassium concentrations in heat-stressed sheep to enhanced aldosterone activity and dehydration-induced electrolyte redistribution. Electrolyte loss through sweat and urine under thermal stress triggers compensatory mechanisms to maintain osmotic balance. This is consistent with Sammad et al. (2020) reported that animals exposed to grazing during hotter hours of the day experienced greater heat stress, which triggered fluid loss and elevated sodium and potassium levels as part of the body's natural response to maintain hydration and electrolyte stability. Furthermore, Périard et al. (2021) observed that during heat stress, animals depend on electrolyte regulation and redistribution as essential strategies to sustain fluid balance and support normal cellular processes. Although calcium and magnesium levels did not differ significantly among groups, they were numerically higher in lambs grazing during the morning hours. This aligns with Hossain et al. (2023), who found that reduced heat exposure during cooler grazing hours helps improve nutrient intake and mineral utilization, supporting better overall mineral balance and physiological adaptation in livestock. Similar observations were reported by Singh et al. (2016), who noted improved calcium and magnesium profiles in animals exposed to lower environmental temperatures. Additionally, Cukic (2023) emphasized that the regulation of calcium in animals is predominantly controlled by nutritional intake and internal physiological processes, rather than external heat, which may clarify the uniform calcium levels across different grazing schedules. Liver function markers, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST), did not show significant differences among the groups. ALT levels remained within normal ranges, indicating no apparent hepatic damage associated with the different grazing schedules. While AST levels were numerically higher in KHG lambs, they also remained within physiological norms and did not reach statistical significance. Aspartate aminotransferase (AST) is a cellular enzyme involved in amino acid metabolism. Its elevated levels in blood under stress conditions often indicate leakage from damaged liver or muscle cells, reflecting tissue injury due to heat-induced oxidative damage. According to Mylostyva et al. (2023), aspartate aminotransferase (AST) serves as a reliable marker of both heat and oxidative stress in livestock, where increased levels often indicate potential damage to liver or muscle tissue. However, the current results suggest that grazing time did not exert substantial hepatic stress, aligning with Sejian et al. (2013), who reported that liver enzyme activity in sheep is not significantly influenced by variations in grazing schedules, provided animals are managed under balanced nutritional and environmental conditions. Similarly, creatinine and blood urea nitrogen (BUN) levels did not differ significantly among the groups. This finding supports the results of Onasanya et al. (2015), who observed that minor management variations, such as grazing time, do not markedly affect creatinine levels unless animals are subjected to severe stress or renal dysfunction. The stability in urea nitrogen levels also corresponds with Sejian et al. (2012), who reported that BUN is more reflective of dietary protein intake and overall metabolic balance than environmental factors alone. The uniformity of dietary provision across groups in the current study likely contributed to the consistent levels of these renal function parameters.

Table 7 Serum biochemical parameters as influenced by grazing management

Parameters		Groups				
		KMG	KHG	KEG	SE	p-Value
Glucose (mg/dL)	BT	51.8 ± 3.03	53.8 ± 3.11	54 ± 3.39	1.423	0.502
	AT	60.6 ± 3.71 ^{ab}	64.8 ± 3.27^{a}	59.4 ± 1.14 ^{b}	2.034	0.039
Total Protein (g/dL)	BT	6.24 ± 0.54	6.14 ± 0.28	6.2 ± 0.46	0.198	0.938
	AT	6.84 ± 0.40 ^{ab}	6.48 ± 0.29 ^{b}	7.14 ± 0.33^{a}	0.155	0.034
Sodium (mEq/L)	BT	142 ± 2.54	141.4 ± 2.07	143.2 ± 2.58	1.080	0.506
	AT	143.4 ± 3.66 ^{b}	147.6 ± 1.14 ^{a}	144.6 ± 2.07 ^{ab}	1.061	0.042
Potassium (mEq/L)	BT	4.26 ± 0.28	4.3 ± 0.29	4.42 ± 0.28	0.129	0.668
	AT	4.36 ± 0.19 ^{b}	4.68 ± 0.21 ^{a}	4.46 ± 0.11 ^{ab}	0.080	0.043

Calcium (mg/dL)	BT	11.8 ± 0.22	12.06 ± 0.51	11.92 ± 0.37	0.174	0.586
	AT	12.24 ± 0.23	11.96 ± 0.24	12.14 ± 0.38	0.131	0.344
Magnesium (mg/dL)	BT	2.36 ± 0.24	2.32 ± 0.19	2.44 ± 0.23	0.099	0.692
	AT	2.6 ± 0.15	2.48 ± 0.14	2.56 ± 0.20	0.077	0.553
Alanine transaminase (ALT units/L)	BT	29.4 ± 2.40	28 ± 2.54	27.8 ± 1.48	0.983	0.477
	AT	30 ± 3.08	33 ± 2.91	29.8 ± 1.48	1.160	0.134
Aspartate transaminase (AST units/L)	BT	71.4 ± 5.59	73.2 ± 7.39	70.6 ± 7.09	3.014	0.825
	AT	91.4 ± 5.59	101.2 ± 10.23	95.4 ± 8.23	3.686	0.209
Creatinine (mg/dL)	BT	1.24 ± 0.16	1.3 ± 0.15	1.32 ± 0.21	0.081	0.775
	AT	1.38 ± 0.16	1.58 ± 0.13	1.42 ± 0.19	0.073	0.168
Urea nitrogen (mg/dL)	BT	11.38 ± 2.12	12.14 ± 1.62	10.9 ± 2.19	0.893	0.624
	AT	11.72 ± 2.07	13.08 ± 1.51	11.24 ± 2.02	0.843	0.313

KMG = Kachhi morning grazing; KHG = Kachhi hot hours grazing; KEG = Kachhi evening grazing; SE = Standard Error; Values denoted by a different superscript letter (a, b) differ significantly ($p<0.05$). BE = Before treatment; AT = After treatment

Conclusion

This study demonstrated that allowing Kachhi lambs to graze during the cooler morning hours resulted in improved physiological, hematological, hormonal, and oxidative stress responses, along with reduced expression of HSP-70 and HSP-90, indicating lower heat stress. These findings highlight the significance of adjusting grazing schedules as a practical and cost-effective strategy to enhance heat tolerance in sheep under hot climates. Future studies should investigate additional management and nutritional interventions to further strengthen heat resilience and improve overall productivity in sheep production systems.

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