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Unveiling the Biochemical, Enzymatic and Hormonal Stress Biomarkers: Effects of Transportation and Lairage on Sheep Welfare and the Meat Quality

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ABSTRACT

The objective of this study was to assess the impact of transportation and lairage on biochemical, enzymatic, hormonal changes, and meat quality in Nellore Jodipi sheep. Blood samples were collected from two groups of sheep, L0 (slaughtered immediately after transportation without rest) and L12 (12 h of lairage) during exsanguination to analyze biochemical (blood glucose, creatinine, and total protein), enzymatic (creatine kinase [CK], lactate dehydrogenase [LDH], aspartate transaminase [AST], and alanine aminotransferase [ALT]), and hormonal (cortisol, triiodothyronine [T3], and thyroxine [T4]) parameters. Meat samples from the *longissimus dorsi* muscle were examined for pH, r-value, instrumental color, water holding capacity (WHC), shear force, drip loss and cooking loss. Lower ($P<0.05$) levels of glucose, creatinine, total protein, LDH, CK, ALT, cortisol, and T4 were noticed in L12. Meat quality parameters indicated higher ($P<0.05$) pH, r value, and a^* values during the post-mortem period in the L0 group compared to L12. However, no difference ($P>0.05$) was observed for cooking loss, or drip loss values between the groups. Current findings suggest that the slaughter of sheep immediately after transportation without lairage results in increased stress levels in animals adversely affecting both welfare and meat quality. From the present study, it is concluded that a minimum of 12 h lairage period will allow sheep to regain normal homeostasis.

Key words: Welfare, Stress Biomarkers, Transportation, Lairage, Sheep

INTRODUCTION

Sheep farming systems often employ the regular practice of transporting animals to facilitate their feeding or slaughter. This transportation process, involving handling, loading, fasting, confinement, and exposure to unfamiliar environments, can induce stressful physiological and behavioural changes in sheep, as reported

in studies by Broom (2003); Cockram et al. (2000) and Grandin (1997). The ability of animals to cope with stress resulting from feed and water withdrawal may vary with age, as observed by Fisher et al. (2008). Transporting farm animals on highways has economic implications, but it also exposes them to various forms of stress, including physical, Physiological, and physiological challenges (Knowles et al., 1998). This stress can negatively impact animal health, well-being, performance, and the final product quality (Aggarwal and Upadhyay, 2013). Proper management during these pre-slaughter stages is crucial for minimizing stress levels effectively. Research efforts should focus on generating comprehensive scientific data to inform standardized regulations at a national level (Cockram, 2007). While some studies have suggested that sheep can tolerate feed and water withdrawal for up to three days (Cole, 1995), the European Union has established maximum journey times and mandatory rest periods for sheep transportation.

Previous studies have primarily examined the detrimental effects of transportation on sheep within specific durations, such as up to 24 h (Knowles et al., 1995), 48 h (Fisher et al., 2010), and 72 h (Horton et al., 1996). Physiological stress experienced during transportation can be reflected in blood components such as cortisol, glucose, and creatine kinase (Fisher et al., 2010). Various physical, physiological, and psychological responses during transportation can negatively impact lamb meat quality (Fuente et al., 2010), including factors like bruising, dehydration, and glycogen depletion in muscles (Knowles et al., 1998). Several authors (Bórnez et al., 2009a; Tadich et al., 2009) have used biomarkers, including plasma concentration of CK, LDH, glucose, and cortisol, to evaluate stress levels induced by transportation in farm animals. These biomarkers can indicate alterations in blood constituents, underscoring their importance in assessing transportation-related stress. Given the above information, further research is necessary to validate the effects of transportation in Nellore jodipi sheep meat quality and physiological responses. The objective of the current study was to compare the blood biochemical, enzymatic, hormonal, and meat quality parameters of sheep that were transported, immediately slaughtered without rest and those which are transported, rested in lairage for 12 h followed by slaughter.

MATERIALS AND METHODS

This investigation was conducted at ICAR- National Meat Research Institute in Hyderabad, and it received approval from the institutional Animal Ethics Committee (IAEC No. 007/NRCM/IAEC-9). Nellore Jodipi sheep, aged 1.5 years with a live weight of ~20 kg, were randomly selected (n=20). These animals were procured from Municipal Slaughterhouse, Chengicherla, Hyderabad as soon as they arrive from a long distance overnight (~12 h) transportation (>500 km) by trucks. They were divided into two groups: L0, which were immediately slaughtered after transportation without rest, and L12, which were rested for 12 h at the lairage with ad-libitum water before slaughter. Both groups of animals were subjected to electrical stunning at a voltage of 110 V, 1A for 5 s (electrodes applied on both sides of the head behind the ears) prior to slaughter. After sticking on a bleeding platform, blood was collected and blood volume was measured after 4 min bleeding and serum was separated. The serum samples were then analyzed for biochemical, enzymatic, and hormonal profiles. Meat samples from the Longissimus dorsi muscle were collected and examined for pH, R-value, and instrumental color (L^*, a^*, and b^*) variations at different post-mortem periods (1 h, 8 h, 12 h, and 24 h). The remaining samples were chilled for 12 h at 4±1 °C, and their meat quality was analysed.

Analytical procedure

Biochemical, enzymatic and hormonal analysis

Ethylene-diamine-tetra acetic acid (EDTA) vials were used for collecting the blood samples. The blood tubes were immediately subjected to centrifugation at 3000 rpm for 15 min, maintaining a temperature of 4 °C. The resulting plasma fraction was divided into smaller portions (aliquots) and stored at -80 °C for further analysis. The blood samples were then examined to determine the levels of various biochemical parameters such as glucose, total protein, and creatinine, alanine aminotransferase (ALT), aspartate transferase (AST), and lactose dehydrogenase (LDH), cortisol, triiodothyronine (T3), and thyroxine (T4). All of the blood samples were analyzed using commercial kits from Everlife CPC Diagnostics, Chennai, India, and an automated chemistry analyzer (Model: TurboChemNeo, CPC Diagnostics) was utilized for this purpose.

pH

The meat sample's pH was assessed at different post-mortem time points at 1, 8, 12, and 24 h. The pH measurement was analysed using a digital pH meter with a pre-standardized electrode (model: HENNA, HI 2216, Europe)

R- value

The R-value was determined following the Honikel and Fischer (1977) protocol for each post-slaughter interval (1, 8, 12, and 24 h).

Instrumental colour

The breast meat samples were assessed for their lightness (L^*), redness (a^*), and yellowness (b^*) at different time intervals after slaughter (1, 8, 12, and 24 h). This measurement was carried out using a colorimeter (Model: CR-20, KONICA MINOLTA, INC., Japan).

Water holding capacity

To determine the water holding capacity (WHC), the method described by Wardlaw et al. (1973) was employed.

Warner-Bratzler shear force

The Warner-Bratzler shear force (WBSF) values were measured using a texturometer (Model H1KF; Tinius Olsen, Redhill, England) equipped with a V-shaped stainless-steel blade set at a 90° angle. The shear force was calculated in Newtons (N) as the cores were sheared perpendicularly to the crosshead at a load range of 75 Newtons (N), with the crosshead speed set at 200 mm/min.

Drip loss and cooking loss

The drip loss was assessed using the method outlined by Honikel and Fischer (1977), with minor modifications. The cooking loss was calculated as the percentage difference in weight before and after cooking.

Statistical analysis

The experiment utilized a fully randomized design with five repetitions (n=20). The statistical analysis was performed using OriginPro software (OriginPro, Version 2023. OriginLab Corporation, Northampton, MA, USA). To examine the effects of lairage (T0 and T12) on biochemical and meat quality characteristics, t-test was employed. Additionally, a two-way analysis of variance was conducted to assess the impact of lairage and post-mortem storage period (1, 8, 12, and 24 h) on pH, R-value, and instrumental color. Subsequently, the Tukey test at a 95% confidence level ($P<0.05$) was applied to determine significant differences among the means.

RESULTS AND DISCUSSION

Biochemical, enzymatic, and hormonal stress biomarkers

The results revealed that the L0 group exhibited higher ($P<0.05$) blood glucose, creatinine, and total protein levels compared to the L12 group (Table 1). The observed increase in plasma total protein levels in the L0 group can be attributed to various factors associated with the physiological response to stress. Stress prompts the release of stress hormones, such as corticosteroids, which stimulate the synthesis of proteins and the mobilization of amino acids from peripheral tissues to the bloodstream (Perai et al., 2015). Similar increased total protein content was observed in the transported group of Imroz rams

Table 1. Blood biochemical parameters in sheep subjected to slaughter immediately after transportation (L0) and slaughtered after 12 h of lairage (L12).

Parameters	L0	L12	P value
Glucose (mg/dl)	100.19±11.27[a]	81.19±0.21[b]	0.043
Creatinine (mg/dl)	1.14±0.05[a]	0.89±0.02[b]	0.001
Total protein (g/dl)	8.42±0.16[a]	7.06±0.36[b]	0.004
Lactate dehydrogenase (LDH) (U/L)	798.97±6.26[a]	238.6±2.6[b]	<0.001
Creatine kinase (CK) (U/L)	147.49±1.55[a]	134.93±3.63[b]	0.005
Aspartate Transferase (AST) (IU/L)	6.06±2.38[a]	7.67±0.47[a]	0.315
Alanine aminotransferase (ALT) (IU/L)	60.63±2.64[a]	26.76±0.55[b]	<0.001
Cortisol (µg/dl)	2.82±0.01[a]	2.37±0.06[b]	<0.001
Triiodothyronine (T3) (ng/dl)	2.09±0.02[a]	1.81±0.32[a]	0.207
Thyroxine (T4) (µg/dl)	3.22±0.06[a]	2.77±0.1[b]	0.002

[a–b]Means ± standard deviation without a common superscript were determined to be significantly different between treatments (columns) n=20

(Ekiz et al., 2012). Moreover, stress-induced inflammation can play a role in raising the levels of acute-phase proteins such as C-reactive protein and fibrinogen, which are a specific group of proteins that respond to tissue injury or inflammation and could be responsible for increasing the levels of total protein in the plasma during times of stress (Cray, 2012). The results obtained in our study for blood glucose levels were consistent with the findings of Zhong et al. (2011) who reported a higher blood glucose level in transported sheep compared to non-transported sheep. Ali et al. (2006) discovered elevated plasma glucose concentrations in transported sheep, attributing this finding to the secondary effects of hypercortisolemia and enhanced glucose production from the liver, indicative of heightened sympatho-adrenal activity resulting from stress. The release of stress hormones such as glucagon and cortisol are a key mechanism in the body's response to stress. These hormones stimulate processes like glycogenolysis and gluconeogenesis, leading to the production and release of glucose into the bloodstream. In our study, we observed significantly higher blood creatinine levels in the L0 group compared to the L12 group. This finding suggests that the transportation process may have contributed to an increased breakdown of muscle tissue in the L0 group, leading to higher levels of creatinine in their blood.

The CK, LDH, and ALT have been employed as indicators to assess trauma, high levels of physical activity, or other forms of damage that may occur during the handling and transportation of farm animals, as noted in studies by Bórnez et al. (2009b); De la Fuente et al. (2010); Elif and Hulya (2013) and Tadich et al. (2009). The comparison between the L0 group and the L12 group revealed significant differences in LDH, CK, and ALT levels. The L0 group displayed significantly higher ($P<0.05$) LDH, CK, and ALT levels compared to the L12 group, however, no significant difference was observed in AST levels between the L0 group and the L12 group (Table 1). Our study findings were in accordance with the results reported by Liste et al. (2011) and Liu et al. (2012), who observed significantly higher levels of CK, ALT, and LDH in sheep that were transported and slaughtered immediately, compared to those underwent a 12 h lairage period.

The results demonstrated significant differences in plasma cortisol and T4 levels between the L0 group and the L12 group. The L12 group exhibited significantly lower plasma cortisol and T4 levels compared to L0. Additionally, no significant change was observed in T3 levels among the groups (Table 1). Cortisol is a hormone released in response to stress, and elevated levels can indicate physiological stress experienced by animals. Our findings align with the study conducted by Leme et al. (2012), which explored the influence of road transport in open or closed compartments on cortisol levels as an indicator of stress in lambs and observed a decrease in cortisol levels in lambs with extended lairage time suggesting a potential reduction in stress levels associated with lairage. Similar higher cortisol levels were reported in the transported group compared to non-transported sheep (Ekiz et al., 2012). The decreased cortisol levels in the L12 group indicate that the rest period allowed for a recovery from the stress of transportation. Moreover, the presence of corticosterone, another stress-related hormone, has been associated with inhibiting the synthesis of both T4 and T3 thyroid hormones. Additionally, the activity of the enzyme responsible for converting T4 to T3 in peripheral tissues may be suppressed under stress conditions. These factors can disrupt the functioning of the hypothalamic-pituitary-thyroid axis, leading to reduced production and secretion of thyroid hormones (Kelly, 2000).

Meat quality parameters

Our study revealed that the L0 group consistently exhibited significantly higher levels ($P<0.05$) of pH from 1 h to 24 h post-mortem compared to the L12 group. Additionally, the pH values were significantly higher ($P<0.05$) in L0 (5.8) compared to L12 (5.7). However, there was a progressive and significant ($P<0.05$) reduction of pH in both treatment groups over time (Figure 1). Our findings are consistent with previous studies that have reported higher ultimate pH values in muscle from restraint-stressed sheep (Apple et al., 1995) and transported goats (Kadim et al., 2006). Li et al. (2018) also observed higher pH values in sheep subjected to transportation without lairage compared to those with a 12- h lairage period and a control group with no transportation or lairage. Similarly, Ekiz et al. (2012) reported higher pH values in transported sheep with 18- h lairage compared to those with a 30-minute lairage at both 0- and 24- h post-mortem. The influence of transport and pre-slaughter management on ultimate pH can vary in different studies. While some studies have found an increase in ultimate pH due to transport and pre-slaughter handling (Vergara et al., 2005). Muscle glycogen content is another important factor influencing meat quality, and transportation distance has been associated with glycogen depletion and higher ultimate pH levels (Zhang et al., 2009). These findings suggest that transportation and pre-slaughter factors can affect the metabolic processes in the muscle, leading to changes in pH and glycogen levels, which in turn influence meat quality.

The study revealed significant differences ($P<0.05$) in the R values between L0 and L12 groups during the

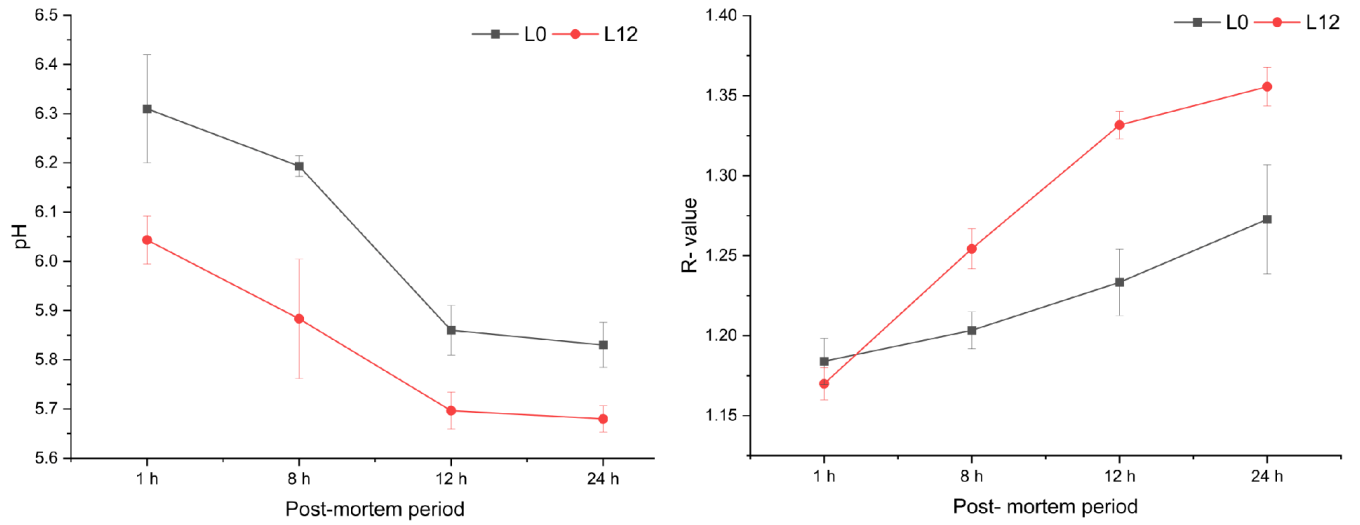

Figure 1. pH and R- value changes in *longissimus dorsi* muscle of sheep between immediate slaughter after transportation and slaughtered after 12 h of lairage during different post-mortem storage periods.

post-mortem period. Specifically, lower R values were observed at 8, 12, and 24 h in the L0 group compared to the L12 group (Figure 2). However, no significant difference (P>0.05) was found at 1 h post-mortem between these groups. Furthermore, there was a progressive increase in R values from 1 h to 24 h in the L12 group, and these changes were statistically significant (P<0.05). On the other hand, within the L0 group, there were no significant differences (P>0.05) between 1 h, 8 h, and 12 h of post-mortem storage. However, there was a significant increase (P<0.05) in R values at 24 h compared to 1 h post-mortem in the L0 group (Figure 1). These findings are consistent with previous research that has investigated the influence of stress on the sympathetic nervous system and its role in eliciting physiological responses in animals (Spinosa et al., 2006). When confronted with stressful conditions, the sympathetic nervous system autonomously releases catecholamines, which activate voltage-gated calcium channels. This activation results in an influx of calcium ions into the sarcoplasm, thereby accelerating the utilization of muscle adenosine triphosphate (ATP) and the process of glycogenolysis. Consequently, there is a rapid decrease in muscle pH (Hussnain et al., 2020). The notable progressive increase in the R-value observed from 1 h to 24 h during post-mortem in both treatment groups indicates the presence of ongoing metabolic activity during this period. These findings suggest that the processes associated with muscle metabolism and the breakdown of ATP and glycogen persist even after the slaughter process, leading to changes in the R-value over time.

A significantly (P<0.05) higher a^* value was observed in L0 during 12 and 24 h of post-mortem. However, no statistically significant differences (P>0.05) were observed in the L^*, a^*, and b^* values between the L0 and L12 groups during 4, 8, 12, and 24- h post-mortem storage periods (Table 2). Our results are consistent with the findings of (Teke et al., 2014), who reported higher a* values in lambs slaughtered immediately after transportation, followed by 2 h and 4 h of lairage. Contrary to our findings, Campo et al. (2010); Ferreira et al. (2006) did not find any significant influence of rest or lairage time on L^* values in cattle. In addition, Bond et al. (2004) observed that the meat color of lambs subjected to exercise stress was darker than that of non-exercise stress lambs. The difference in meat lightness between stall-raised and pasture-raised lambs, as noted by Priolo et al. (2002), may be partially attributed to the variation in ultimate pH, as higher pH meats tend to have a darker colour.

A significantly higher (P<0.05) WHC was observed in L0 compared to L12 group (Table 3). Our results are in line with the findings of Ekiz et al. (2012), who reported significantly higher WHC in sheep subjected to transportation and 30 min of lairage compared to those subjected to transportation and 18 h of lairage, as well as non-transported groups. This indicates that the duration of lairage after transportation can have an impact on the WHC of the meat. The relationship between WHC and muscle pH is well-established. Fuente et al. (2010) noted that proteins in muscle are able to bind more strongly with water at higher pH levels, resulting in improved WHC and less free water in the meat. Similarly, Kadim et al. (2009) reported reduced WHC in meat samples with low ultimate pH values in sheep. Consistent with these findings, Argüello et al. (2005) found a negative correlation between ultimate

Table 2. Instrumental color variations during different post-mortem storage periods of *longissimus dorsi* muscle in sheep subjected to slaughter immediately after transportation (L0) and slaughtered after 12 h of lairage (L12).

	Treatment Groups	4 h	8 h	12 h	24 h	SEM
*L**	L0	38.82±1.65	40.9±0.97	40.37±1.48	40.8±2.3	0.308
	L12	38.33±1.55	39.92±0.02	40.3±1.57	39.43±1.1	
*a**	L0	10.83±0.03^{ab}	11.98±0.52^{ab}	12.18±0.42^{a}	12.39±1.13^{a}	0.172
	L12	10.77±0.64^{ab}	11.07±0.44^{ab}	11.08±0.55^{ab}	10.5±0.3^{b}	
*b**	L0	6.35±0.73	8.12±1.82	8.52±3.05	8.63±2.9	0.366
	L12	6.3±0.7	8.87±0.63	8.08±0.98	8.88±1.32	

$^{a–b}$Means ± standard deviation without a common superscript were determined to be significantly different, separately for L, a* and b* values. n=20

Table 3. Physiochemical changes of *longissimus dorsi* muscle in sheep subjected to slaughter immediately after transportation (L0) and slaughtered after 12 h of lairage (L12).

Parameters	L0	L12	P- value
WHC (%)	13.28±0.44^{a}	11.17±0.28^{b}	0.002
Shear force value (N)	11.85±0.28^{a}	8.25±0.35^{b}	<0.001
Drip loss (%)	1.61±0.12	1.71±0.09	0.286
Cooking loss (%)	29.17±1.22	28.48±1.13	0.512

$^{a–b}$Means ± standard deviation without a common superscript were determined to be significantly different.
[2]WHC, Water holding capacity values were expressed in percentage and determined at 8 h post-mortem. n=20

pH value and the amount of juice expelled, indicating that higher pH meats tend to have better water holding capacity. Additionally, Apple et al. (1995) observed that meat from stressed lambs had higher ultimate pH values compared to non-stressed lambs.

Significantly lower (P<0.05) shear force values (higher tenderness) were observed in L12 compared to L0 treatment group (Table 3). The potential reason for tougher meat in stressed sheep, characterized by sarcomere shortening, was suggested by Kadim et al. (2006). Increasing the lairage duration from 30 min (TS-L30 min treatment) to 18 h (TS-L18 h treatment) in transported lambs was found to result in a noticeable improvement in meat tenderness, as indicated by a decrease in Warner Bratzler shear force value (Ekiz et al., 2012). Similar findings were reported by Campo et al. (2010), who observed that meat from steers subjected to a long lairage period (15 h) displayed lower Warner Bratzler shear force values compared to those with a short lairage period (3 h). The authors noted that a short lairage time, without adequate rest, led to increased ultimate pH and shear force values.

No significant difference (P<0.05) was observed in drip loss between L0 and L12 groups (Table 3). This finding implies that the resting period of 12 h did not result in a significant change in drip loss compared to immediate slaughter after transportation. Our results of drip loss were in accordance with the results of Ekiz et al. (2012), who found no significant difference among transported and 18 h lairage, transported and 30 min lairage and non-transported groups in sheep. No significant (P<0.05) difference was observed in cooking loss between To and T12 treatment groups. Our findings aligned with Bulent et al. (2018) findings, which demonstrated no significant disparity in cooking loss among lambs slaughtered immediately after transportation, those with 2 h of lairage, and those with 4 hours of lairage. Similarly, Li et al. (2018) observed no significant impact on cooking loss when comparing groups that underwent transportation and a 12 h lairage period to non-transported groups.

CONCLUSION

The findings of the present study suggest that slaughtering sheep immediately after transportation without any resting can induce stress and compromise their welfare. This is evidenced by the increased levels of glucose, creatinine, total protein, LDH, CK, ALT, cortisol, and T4. Lambs that were transported and slaughtered after a 12-h resting period exhibited tender meat, with lower Warner Bratzler shear force values and increased water holding capacity. Consequently, the results of this study regarding meat tenderness, redness value, and water holding capacity suggest that slaughtering lambs without any lairage period may result in lower meat quality compared to lambs slaughtered after an overnight resting duration. Therefore, from a meat quality standpoint, it is recommended to slaughter lambs after a minimum of a 12-h resting period.

COMPETING INTEREST

The authors declare no conflicts of interest regarding the materials presented in this paper.

ETHICS STATEMENT

The animal experiments conducted in this study were carried out in adherence to the approved protocols by the Institutional Animal Ethics Committee of ICAR-National Meat Research Institute (IAEC No. 007/NRCM/IAEC-9). All animal procedures strictly adhered to the regulations and guidelines set forth by this committee, ensuring the welfare and minimizing the suffering of the animals involved.

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