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Effects of exposure to slaughter ambient on catecholamines, β-endorphin, plasma enzymes, apoptotic index and shear-force of *Longissimus thoracis et lumborum* muscle in goats

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ABSTRACT

The present study was undertaken to evaluate the effect of exposure to the slaughter ambient on catecholamines, β-endorphin, plasma enzymes, and apoptotic index and shear-force of *Longissimus thoracis et lumborum* (LTL) in goats. A total of 18 goats (Boer cross bucks, 1 year age, 27.50 ±1.5 kg live weight) used in the study were divided into three groups viz. goats were slaughtered alone (Control), goats were exposed to psychological stress by exposure to the slaughter ambient (E) and goats were slaughtered in front of E goats (S). The apoptotic index of LTL muscle cells was assessed at pre-rigor state. The dressed carcass was kept in a chiller (4±1°C) for 5 days and shear force values of LTL muscle were assessed on 0-day, 1 day (24 h), and 5-day intervals. The exposure to slaughter ambient caused a significant ($p<0.05$) increase in lactate dehydrogenase (LDH), nor-adrenaline, and β-endorphin levels. The apoptotic index of the E samples was recorded as significantly ($p<0.05$) higher than control and S samples. The apoptotic index of the S samples was significantly ($p<0.05$) higher than the C samples. As for the shear force, the E samples exhibited lower values ($p>0.05$) than the C and S samples. Thus, the present study highlights the influence of preslaughter exposure to slaughter ambient on the tenderness of LTL muscle in goats during post-mortem aging.

Keywords: Apoptotic index, β-endorphin, Catecholamines, Preslaughter stress, Shear-force, Slaughter ambient

INTRODUCTION

Proper handling of animals before slaughter is very crucial to improve animal welfare compliance and meat quality. With the increasing awareness and consumer preference for products sourced from proper animal welfare compliance procedures throughout the chain of production comprising from livestock rearing to slaughtering (Kumar *et al*., 2022).

Under stressful conditions, the activation of the sympatho-adrenal (SPA) and hypothalamic-pituitary-adrenal axis (HPA) system resulted in the release of catecholamines (adrenaline and nor -adrenaline), which increases the catabolism/ energy-producing metabolisms for fight or flight response (Kumar *et al*., 2022). This causes changes in the physiological and behavioral parameters in animals, which provide good indicators of stressful conditions in animals (Naldurtiker *et al*., 2020; Terlouw and Bourguet, 2022). The higher physical activity under stressful conditions causes damage to muscles and increases their permeability. This resulted in an increased concentration of LDH and creatine kinase (CK) in blood plasma (Jama *et al*., 2016).

Preslaughter stress severely affects the glycogen availability and metabolic rate in muscle, thereby leading to undesirable post-mortem pH changes affecting meat quality (Terlouw *et al*., 2021). Preslaughter stress immediately before slaughter such as intense physical activity, poor restraints, use of electric prods increases the muscular metabolic activity during slaughtering as well as also during pre- and post-rigor state (Jia *et al*., 2007). The resultant high temperature and low ultimate pH (pHu) cause the denaturation of proteins leading to low water holding capacity, higher drip loss, affecting the color, and tenderness/ toughness of meat due to the precipitation of these proteins during cooking (Barbut *et al*., 2008). Other factors that affect meat quality are several preslaughter stress-mediated interconnected pathways of muscle contraction, muscle structure, energy metabolisms, and chaperone-mediated protein folding (Gagaoua *et al*., 2021).

Meat proteomics helps in understanding the complex mechanisms of reactions and changes undergoing in muscle proteins (transcription and translational changes, protein folding, phosphorylation, alteration of cell membrane permeability, myoglobin oxidation) that affect the post-mortem changes and meat quality (Franco *et al*., 2015; Huang *et al*., 2020). Autophagy biomarkers such as Beclin 1, the ratio of LC3-II/ LC3-I, and antioxidant potential were affected by preslaughter stress and could be used in predicting preslaughter stress in animals within the first 24 h post-mortem (Rubio-González *et al*., 2015). The oxidative stress caused by preslaughter stressors was observed to increase the release of calcium ions, caspase-3 and caspase-9 activities, and cytochrome-C. These changes initiate an apoptotic cascade, thereby affecting tenderness in yak meat (Wang *et al*., 2018).

Apoptosis refers to programmed cell death and is a natural process affecting tissue development and homeostasis (Loo, 2011). It is the first step in the conversion of muscle to meat and subsequent changes are induced by various structural changes with caspase enzymes playing a crucial role in initiating apoptosis (Ouali *et al*., 2007). Apoptosis is associated with the cleavage of cellular components, cytoskeletal proteins, loss of membrane integrity, shrinkage, and DNA fragmentation, consequently affecting meat quality parameters (Ouali *et al*., 2007). Higher caspase isoform expressions during post-mortem aging were reported to correlate with lowering shear force in various skeletal muscles of pork (Kemp *et al*., 2006) and in bovine muscle (Cao *et al*., 2010). Apoptotic cells could be identified by TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labelling) assay, DNA laddering, or comet assay, with TUNEL assay regarded as the most sensitive, rapid, accurate, quantitative, and universally accepted method (Majtnerová and Roušar, 2018). It is based on labelling the 3’ OH end with a specific 3’ OH-end specific terminal deoxynucleotidyl transferase enzyme.

There are studies evaluating apoptosis with post-mortem conditioning and meat quality (Cao *et al*., 2010; Ouali *et al*., 2007; Tian *et al*., 2022; Zhang *et al*., 2013). There is a need to study apoptosis in relation to pre-slaughter stress responses to get a deep insight into meat proteomics and its effect on meat tenderization. Thus, the present study was undertaken to evaluate the effect of preslaughter handling on the catecholamines, β-endorphin, plasma enzymes and apoptotic nuclei in the *Longissimus thoracis et lum-*

borum muscle of goats and meat tenderization.

MATERIALS AND METHODS

Ethical Approval

The present study was approved by animal ethics guidelines of the Research Policy of Universiti Putra Malaysia as per the Institutional Animals Care and Use Committee (Approval No.: UPM/IACUC/AUP-R003/2022 dated 27 May 2022).

Animals

A total of 18 bucks (Boer cross, 8-12 months of age, 27.50 ±1.5 kg liveweight) were purchased from Global Field Trading, Jalan 9/6 Seksyen 9, 43650 Bandar Baru, Bangi, Malaysia and housed at small ruminant housing facility at the Institute of Tropical Agriculture and Food Security in Universiti Putra Malaysia (latitude $2^0$59'06.5" N and longitude $101^0$43'40.7" E) for 2 weeks for acclimatization. During their stay, bucks were dewormed and fed twice daily (morning-chopped fresh Napier grass and evening pellets) and *ad libitum* potable water. Various physiological parameters (feeding pattern, breathing, rectal temperature, heart rate, and gait) were closely observed and recorded on the animal monitoring sheet (Kumar *et al*., 2024).

Before the start of the experiment, the healthy bucks were road transported in a covered lorry (capacity 1.5 tons) to the research slaughterhouse of the Department of Animal Science, Faculty of Agriculture, UPM (258059.000" N; 10144006.400" E) in the evening hours (3.30-4.30 pm) covering 2.0 km within 30 - 40 mins (in loading, transit, and unloading). The bucks were rested overnight in the lairage with the provision of *ad libitum* water.

Experimental Design

To replicate the practice of slaughter during religious sacrifice in developing countries, the goats were exposed to the slaughter environment through their auditory, olfactory, and visual senses. The distance between the goat visualizing the slaughter process and the slaughtered goat was kept at the same distance (approx. 2 m) throughout the study (Kumar *et al*., 2023a). The goats were divided into three groups viz., C group: Control group where a goat was slaughtered alone, without the presence of any other goats; E group: Goats exposed to the slaughter environment by witnessing the slaughter of one goat from the S group; S group: Goats slaughtered in front of one E-group goat. The goats were Halal slaughtered by transverse severance of the carotid arteries and jugular veins by the trained slaughterman following the standard protocols outlined in the MS 1500:2009 (Department of Standards Malaysia, 2009).

Immediately after slaughter, goat carcasses were dressed and kept in the chiller at 4±1 °C for post-mortem aging for 5 days. The *Longissimus thoracis et lumborum* (LTL) muscle, due to being the largest and index muscle for meat quality parameters, was used to evaluate apoptotic index and shear force value.

Blood Collection

Blood collection was performed by the trained technical staff from the external jugular vein by using 21-gauge needles connected to a 10 mL clot activator (BD Vacutainer®, Plymouth, UK) ethylenediaminetetraacetic acid (EDTA) tubes as per Kumar *et al*. (2024). The tubes containing collected blood samples were kept slat in a box containing crushed ice for 1 h, followed by refrigerated centrifugation (Eppendorf Centrifuge 5810) at 3500 g for 15 min at 4 °C. The plasma obtained was stored in a deep freezer (Sanyo Electric Co Ltd, UK) at -80 °C, until subsequent hormonal analysis.

Blood Hormones and Enzymes Assays

Plasma concentrations of catecholamines and β-endorphin were determined using the highly sensitive adrenaline (BA E-4100), noradrenaline (BA E-4200), and β-endorphin (QY-E140008) enzyme-linked immunoassay kits (QAYEE-BIO-Technology Co. Ltd, Shanghai, China). LDH and CK plasma concentrations were assessed using LDH and CK kits (Bioassay System, D2DH-100, CA, USA). The manufacturer's instructions for the kits were followed during the plasma hormone and enzyme analysis.

Terminal Deoxynucleotidyl Transferase dUTP Nick-end Labelling (TUNEL) Assay

TUNEL assay was utilized to detect apoptotic cells in muscle in this investigation (Didenko, 2011). This method is based on the

detection of DNA fragmentation in apoptotic cells. DNA polymerase and terminal deoxynucleotidyl transferase (TdT) labeled the broken DNA strand. This was accomplished with a commercial In-Situ Cell Detection Kit (Roche Molecular Biochemicals, Mannheim, Germany).

The LTL muscle tissue slides were prepared as per the following process followed by Ansari *et al*. (1993). After fixing muscle tissues in 40% buffered formaldehyde, they were embedded in paraffin. The tissue was cut to approximately 5 µm and mounted on glass. After dewaxing the tissue sections at 60 °C for 30 minutes, they were dipped in xylene and ethanol (100%, 95%, 90%, 80%, 70%). It was then deparaffinized and air-dried. Before staining with the TUNEL reaction, the slides were permeabilized with a Triton x-100 and sodium citrate solution and washed with PBS. Afterward, the slides were incubated at 37 °C for 60 min in a dark, humid environment. TUNEL-positive cells were counted using a fluorescent microscope a Carl-Zeiss (Axiovert A1) at a magnification of 20x. Green-fluorescent cells were regarded as TUNEL positive and cells with a red fluorescence (PI stain) were regarded as TUNEL negative. Ten randomly chosen microscopic fields were used to detect TUNEL-positive cells on each slide**.** The apoptotic index was measured for each sample as per formula of Wang *et al*. (2014):

Apoptotic index= {(total count of TUNEL-positive cells)/ (total count of positive cells)} x100

Determination of Shear Force Values

Shear force was assessed by using the Volodkovitch bite jaw shear force test attached to a texture analyzer (TA-XT2i, Stable Micro System, United Kingdom). The cooked muscle samples were cut into 1cm x 1cm x 3cm cubes with the help of a V-shaped shear blade. The samples were sheared perpendicular to the fiber direction. The analysis was based on the mechanical force (kg) required to shear the muscle fibers of a cooked meat sample.

Statistical Analysis

The data generated in the study was examined for normal distribution by the Shapiro–Wilk test using SPSS Statistics Version 20 software (IBM Corporation, New York, USA). Data were presented as mean along with standard error values by using Analysis of Variance (ANOVA). Where significant findings were reported, Duncan's multiple range test was used to compare means (n = 6). A p-value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Catecholamines

Exposure to slaughter ambient affects the release of catecholamines in goats (Table 1). The adrenaline concentration was recorded as the highest in the E group slaughtered after exposure to slaughter ambient and the lowest in the Control group slaughtered alone. However, the adrenaline concertation was recorded as non-significant ($p>0.05$) among the groups. Further, the nor-adrenaline concentration was recorded as significantly higher ($p<0.05$) for the E group as compared to the nor-adrenaline concentration in the Control group. The nor-adrenaline levels in the S and E groups did not have significant ($p>0.05$) differences and were observed to be comparable.

Table 1. Effect of Exposure to Slaughter Ambient on Catecholamines, B-Endorphin, Creatine Kinase, and Lactate Dehydrogenase in Goats

Parameters	Control	S	E	P value
Creatine Kinase (IU/L)	195.70±6.27	219.64±52.62	225.10±42.51	0.854
Lactate dehydrogenase (IU/L)	322.26±5.56a	379.62±29.52ab	407.76±15.05b	0.021
Adrenaline (pg/mL)	69.08±4.74	77.82±5.24	80.98±7.30	0.357
Nor-adrenaline (pg/mL)	140.33±1.03a	170.98±5.44b	174.96±5.61b	<0.001
β-endorphin (pg/mL)	58.73±2.32a	58.34±1.88a	68.12±1.11b	0.003

*Values are mean ±standard error with different superscripts small letters a, b, c--within a row differ significantly ($p<0.05$), Control-goat slaughtered alone, S- goats slaughtered in front of E, E- goats slaughtered after exposure to slaughter ambient, n=6, level of significance $p<0.05$

The higher catecholamine concertation in the E group could be due to higher psychological stress and fear perception in the animals upon exposure to slaughter ambient. The electroencephalogram spectrum, behavioral and physiological responses in the E goats further confirmed this (Kumar et al., 2023a). The higher catecholamines in the S group could be due to the higher psychological stress of handling during slaughter. The pheromones released in the blood of animals could also cause stress in the E goats (Grandin and Vogel, 2011). Higher electrical activity of Cerebro cortical neurons and physiological responses have also been reported in goats during exposure to slaughter ambient (Kumar *et al*., 2023a).

β-Endorphin

The β-endorphin levels were recorded significantly ($p<0.05$) higher for the E group as compared to Control and S goats (Table 1). The hypothalamus and pituitary glands release the β-endorphins under stressful and painful conditions to modulate pain and create a feeling of general well-being. Thus, higher levels of β-endorphin are associated with stressful and painful conditions (Jain *et al*., 2019). However, a higher level of β-endorphin in livestock was reported to correlate with stress rather than pain (Terlouw and Bourguet, 2022). A rapid influx of beta-endorphin (5-10 times) in male rats upon psychological stress of loud noise (40-60 dB sound) (Xiong *et al*., 2019). However, as the blood circulatory β-endorphin has yet to be established to reach the central nervous system, thereby plasma β-endorphin is widely correlated with stress rather than pain perception (Bruehl *et al*., 2012).

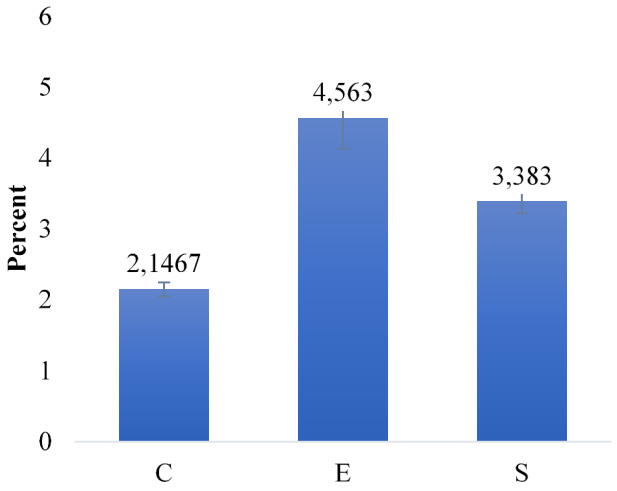
Creatine Kinase (CK) and Lactate Dehydrogenase (LDH)

The CK and LDH concentration in blood plasma provides a good indicator of physical activity, stress, and muscle damage (Table 1). In the present study, CK levels were recorded with a non-significant ($p>0.05$) difference among the groups.. The LDH was recorded significantly ($p<0.05$) higher in the E group as compared to the control and the S group. The LDH levels were comparable between the control and the S group. The higher levels of plasma CK and LDH levels in the E goats, as recorded in the present study, could be due to the high level of stress and physical activity in goats during exposure to slaughter ambient. Under the stress condition, animals undergo higher muscular activity, thereby increasing cell membrane permeability and damage the muscle cells (Adenkola *et al*., 2011). A significant increase in the CK concentration was observed in goats in lairage after transportation stress and after slaughter (Othman *et al*., 2021). The plasma LDH concentration of goats was also reported to have a significant ($p<0.05$) increase due to slaughter stress (Sabow *et al*., 2019). Various preslaughter handling operations could cause stress and muscle injury in animals, leading to increase blood CK and LDH levels (Ekiz *et al*., 2012). In addition, the physical exertion in the lairage could also contribute to the higher plasma CK concentrations (Fàbrega *et al*., 2002).

Apoptotic Index

Apoptosis is associated with the cleavage of cellular components, cytoskeletal proteins, loss of membrane integrity, shrinkage, and DNA fragmentation, consequently affecting meat quality parameters (Ouali *et al*., 2007). The apoptosis is also affected by the lysosomal enzymes, calpains, cathepsins, calpastatin, proteasomes, antioxidants, stress proteins, and other molecules (Park *et al*., 2010).

The apoptotic index of the control sample was recorded as significantly lower ($p<0.05$) than the E and S samples (Figure 1). The E samples recorded the highest apoptotic index value (4.563±0.43) among all three samples followed by S samples (3.383±0.16) and control samples (2.147±0.10). The apoptotic index of E samples was significantly ($p<0.05$) higher than S samples, which in turn exhibited significantly ($p<0.05$) higher apoptotic index than control samples. This could be correlated with higher stress levels in these animals upon exposure to slaughter ambient as evidence from the higher level of catecholamines recorded in Table 1. Various preslaughter stress factors were reported to increase the stress levels in animals, resulting in a higher apoptotic index. A significant increase in the apoptotic index in cattle under preslaughter transportation stress (short distance 450 km in 9 h vs. long distance 850 km in 17 h) and stocking density (200, 400, and 600 kg/m^2) were



(C-control slaughtered alone, E- goats slaughtered after exposure to slaughter ambient, S- goats slaughtered in front of E, n=6)

Figure 1. Apoptotic index of *Longissimus thoracis et lumborum* muscle affected by preslaughter stress of exposure to slaughter ambient in goats

Table 2. Shear force value (kg) of *Longissimus thoracis et lumborum* Muscle of Goat During Post-Mortem Aging Affected by Preslaughter Stress of Exposure to Slaughter Environment

Treatment	0 days	1 day	5 day	P value
C	1.993±0.019^{c}	1.659±0.033^{b}	1.370±0.013^{a}	<0.001
E	1.970±0.020^{c}	1.623±0.033^{b}	1.312±0.024^{a}	<0.001
S	1.992±0.029^{c}	1.649±0.043^{b}	1.343±0.019^{a}	<0.001
P value	0.740	0.775	0.131	

Values are mean ±standard error with different superscripts within a row differ significantly ($p<0.05$), C-C-control slaughtered alone, E- goats slaughtered after exposure to slaughter ambient, S- goats slaughtered in front of E, n=6, level of significance $p<0.05$

reported by Abubakar (2022). Similarly, stress due to heat was observed to increase autophagy in immature porcine Sertoli cells (Bao *et al*., 2017).

The oxidative stress caused by preslaughter handling was observed increasing the release of calcium ions, caspase-3 and caspase-9 activities, and cytochrome-C. These changes initiate an apoptotic cascade, thereby affecting tenderness in yak meat (Wang *et al*., 2018). Further, exposure to preslaughter stress was reported to induce apoptosis and increase the degradation of actin due to the release of cytochrome C and caspase by mitochondria immediately after death (Boudida *et al*., 2015).

Meat Tenderness

Meat tenderness was measured in terms of shear-force value during post-mortem aging of the LTL muscles of goats (Table 2). Within groups, the shear force value of LTL muscles of goats during post-mortem aging was not altered significantly ($p>0.05$). However, the shear force value of E samples was recorded the lowest, and control samples showed the highest shear force value (E>S>C). A significant ($p<0.05$) decrease

in the shear force value for all samples was noted with the advancement of aging duration. The changes in the shear force value in the LTL samples during post-mortem aging could be due to various changes leading from muscle to meat. The lower shear force value in E could be due to higher proteolytic changes. However, the non-significant changes in the shear force value in the present study could be due to keeping the exposure to the slaughter environment minimal (approx. 4-6 min) to minimize the stress levels.

Overall, meat tenderness is affected by the degradation of muscle myofibrils by proteolysis during aging along with sarcomere length, collagen and cross-linking, marbling, and denaturation of meat proteins during heating/ cooking (Purslow *et al*., 2021). Higher caspase isoform expressions during post-mortem aging were reported to correlate with lowering shear force in various skeletal muscles of pork (Kemp *et al*., 2006) and in bovine muscle (Cao *et al*., 2010). Increased intercellular space, shrinkage of cells immediately after bleeding, and structural alteration leading to the dismantling of muscle cells due to apoptosis were proposed to affect the tenderization of meat (Ouali *et al*., 2007). Similarly, a correlation between higher stress and higher apoptosis index affecting the tenderization of carp meat was also reported by Boudida *et al*. (2015).

CONCLUSION

The exposure to slaughter ambient caused a significant increase in lactate dehydrogenase, nor -adrenaline, and β-endorphin levels. The present study correlates the higher psychological stress in goats to a higher LTL muscle cell apoptotic index and subsequent changes in tenderization during post-mortem aging. The goat slaughtered alone had a significantly ($p<0.05$) lower apoptotic index as compared to other goats slaughtered after exposing to a slaughter environment and also goats slaughtered in the presence of other goats.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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