

CO₂ stunning improved the meat quality by regulating postmortem muscle cell death

Xiao Qiu, Xinrui Mao, Kai Shan and Chunbao Li* 

State Key Laboratory of Meat Quality Control and Cultured Meat Development, Ministry of Science and Technology (MOST); Key Laboratory of Meat Processing, Ministry of Agriculture and Rural Affairs (MARA); Jiangsu Collaborative Center of Meat Production, Processing and Quality Control; College of Food Science and Technology, Nanjing Agricultural University, Nanjing, Jiangsu 211800, China

* Corresponding author, E-mail: chunbao.li@njau.edu.cn

Abstract

The incidence of abnormal meat poses a significant challenge in the meat industry, where stunning serves as a crucial intervention to alleviate pre-slaughter stress and improve meat quality. The underlying mechanisms through which stunning techniques contribute to the divergence in postmortem pork quality are yet to be fully elucidated. This study reveals that the modality of cellular demise in postmortem muscle tissue is modulated by the intensity of pre-slaughter stress and is a determinant of postmortem meat tenderness. The study also investigated the effects of electrical stunning and carbon dioxide stunning on pork quality in Duroc × Landrace × Yorkshire (DYL) pigs. Energy metabolism, muscle fiber structure, and indicators of cell death were measured. The results showed that compared to electrical stunning, carbon dioxide stunning alleviated the depletion of glycogen and ATP, resulting in a lower accumulation of lactic acid. This process induced apoptosis and activated caspase, thereby improving tenderness: shear force values decreased significantly from 47.02 to 42.31 N ($p < 0.05$). ATP treatment caused the reactivation of caspase and increased meat tenderness. This study confirms the efficacy of carbon dioxide stunning in enhancing meat tenderness and provides initial insights into the underlying mechanisms involving energy metabolism and caspase-dependent pathways.

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Introduction

Stunning is a critical step in pig slaughtering aimed at restricting movement, facilitating subsequent hanging and bleeding, improving slaughter efficiency, and reducing animal pain. However, the most commonly used method in current production, electrical stunning, can cause carcass damage and the occurrence of PSE (Pale, Soft, Exudative) meat, severely impacting economic benefits^[1]. Improper pre-slaughter stunning can adversely affect meat color, tenderness, and water-holding capacity (WHC), significantly reducing meat quality^[2]. Carbon dioxide stunning is a technique that induces unconsciousness via exposure to hypercapnic conditions. The mechanism involves cerebral acidosis triggered by elevated CO₂ levels, which suppresses neuronal activity. Compared to alternative methods, CO₂ stunning offers distinct advantages: reduced physiological stress responses and absence of physical trauma to carcasses. However, its widespread implementation in China remains limited due to prohibitive infrastructure costs. Therefore, it is of significant importance to explore the effects and mechanisms of stunning methods on pork quality and to promote superior stunning techniques.

Meat is primarily composed of muscle cells, and the physiological activities of these cells postmortem significantly influence the final quality formation. How do these two stunning methods affect postmortem cellular activities, and through what pathways do they impact pork quality? Firstly, the hypoxia-induced by carbon dioxide stunning and the convulsions caused by electrical stunning may lead to differences in cellular energy metabolism. The intense pre-slaughter stress and muscle contractions induced by electrical stunning can lead to rapid glycogen depletion in muscles before slaughter, resulting in lactic acid accumulation, pH reduction, protein denaturation, and, ultimately, a decline in meat quality^[3]. On the

other hand, the mode of cell death in postmortem muscle cells is a noteworthy factor. In postmortem muscle, cell death triggered by hypoxia, low temperature, and nutrient deprivation mediates the critical transformation of muscle into edible meat. Several types of programmed cell death have been identified, including apoptosis, necroptosis, autophagy, pyroptosis, and ferroptosis. Different modes of cell death occur under varying conditions and may activate proteases. For instance, apoptosis activates caspases, while autophagy releases cathepsins. Key endogenous enzymes involved in postmortem meat tenderization include calpains, caspases, cathepsins, and proteasomes^[4,5]. Liu et al. found that ferroptosis negatively affects the water-holding capacity of beef^[6]. Similarly, Zhang et al. demonstrated through TUNEL staining that skeletal muscle apoptosis is significantly correlated with meat color, tenderness, and water-holding capacity^[7]. The underlying mechanism may involve varying degrees of protease activation associated with different cell death modes. However, the differences in postmortem cell death and their impact on pork quality under different stunning methods have not yet been studied.

The objectives of this study were to investigate the effects of these two stunning methods on the meat quality of pig *longissimus dorsi* muscle and to elucidate the underlying mechanisms by measuring energy metabolites, muscle fiber structure, cell death indicators, and protease activity.

Materials and methods

Animal stunning and sampling

Twenty-four castrated Duroc × Landrace × Yorkshire (DLY) pigs (100 ± 10 kg) were selected. Among them, 12 pigs were stunned together in a carbon dioxide chamber (95% CO₂, 90 s) and

designated as the carbon dioxide stunning group (CS). The remaining 12 pigs were hung and stunned using a handheld electrical stunning device (120 V, 5 s) and labeled as the electrical stunning group (ES). After stunning, the pigs were thoracically stuck and hung for bleeding within 5 min. Blood samples (10 mL) were collected and centrifuged at $3,000 \times g$ for 10 min. The resulting serum was stored at -20°C for further analysis. Approximately 45 min postmortem, the longissimus dorsi muscles between the 10th and 12th ribs were excised and stored at 4°C . Meat samples (approximately 2 g) were collected at 1, 5, 10, and 24 h postmortem and stored at -80°C for subsequent use.

To validate the impact of ATP on meat quality, an additional 12 castrated pigs (100 ± 10 kg) were subjected to electrical stunning followed by conventional slaughtering. After slaughter, the longissimus dorsi muscle was removed and cut into 2 cm thick steaks (approximately 50 g each). These steaks were divided into two groups: one group was injected with 1 mL of 1.0 mmol/mL adenosine triphosphate disodium solution and labeled as the ATP injection group (AI), while the other group was injected with 1 mL of physiological saline and designated as the negative control group (NC). The steaks were then stored at 4°C . At 0, 1, 5, 10, and 24 h after injection, meat samples were frozen and stored at -80°C for further analysis.

Meat quality measurement

Meat samples ($5\text{ cm} \times 3\text{ cm} \times 2\text{ cm}$) were stored at 4°C until 24 h postmortem. The initial weight was recorded as W_1 . The samples were then packed in retort pouches and heated in a 72°C water bath until the center temperature reached 70°C . After cooking, the samples were cooled in tap water to room temperature (23°C). Surface moisture was gently wiped off, and the samples were weighed again as W_2 . Cooking loss was calculated using the following formula:

$$\text{Cooking loss (\%)} = \frac{W_1 - W_2}{W_1} \times 100$$

Subsequently, the meat samples were cut along the muscle fiber direction into $2\text{ cm} \times 1\text{ cm} \times 1\text{ cm}$ cubes. Shear force was measured using a C-LM3B meter by cutting vertically to the muscle fibers, and the values were recorded.

For drip loss measurement, meat samples were cut into $5\text{ cm} \times 3\text{ cm} \times 2\text{ cm}$ blocks along the muscle fiber direction and weighed as W_3 . Each block was hung by a hook inside an inflated plastic bag and stored at 4°C for 24 h. After storage, the samples were weighed again as W_4 . Drip loss was calculated as follows:

$$\text{Drip loss (\%)} = \frac{W_3 - W_4}{W_3} \times 100$$

The pH of meat samples was measured at 1, 5, 10, and 24 h postmortem using a portable pH meter (Testo 205, Testo, Lenzkirch, Germany). Measurements were performed in triplicate for biological replicates.

Meat color (L^* , a^* , and b^*) was determined using a colorimeter (CR-400, Konica Minolta, Japan) equipped with a D65 standard light source, an 8 mm measurement aperture, and a 10° standard viewing angle. Meat samples were ensured to have a thickness of at least 2.0 cm. Prior to measurement, the colorimeter was calibrated using a standard white porcelain plate at room temperature. Calibration was considered complete when the difference between the measured and standard values did not exceed 0.1%. During measurement, the colorimeter lens was placed perpendicular to the meat surface and tightly pressed against it to prevent light leakage. Care was taken to avoid areas with visible fat or connective tissue.

H&E staining observation

The pork samples were fixed in 4% paraformaldehyde fixative for 48 h and subsequently embedded in paraffin. The paraffin-embedded samples were sectioned into $6\text{ }\mu\text{m}$ slices using a microtome. The sections were deparaffinized in a dewaxing solution (G1128, Servicebio, Hubei, China) for 40 min, followed by sequential immersion in anhydrous ethanol I for 5 min, anhydrous ethanol II for 5 min, and 75% alcohol for 5 min. After dehydration, the sections were rehydrated in ultrapure water.

Tissue sections were stained using a Hematoxylin and Eosin (H&E) HD constant dye kit (G1076, Servicebio, Hubei, China). Following staining, the sections were sequentially placed in anhydrous ethanol three times (2 min each), 2-butanol twice (2 min each), and xylene twice (2 min each) for transparency. Finally, the sections were sealed with neutral gum.

The microstructure of the samples was observed, and images were captured using a microscope (Axio Scope A1, Zeiss, Germany). The captured images were analyzed using ImageJ software.

TUNEL staining

The paraffin sections were deparaffinized three times with xylene (10 min each) and then dehydrated three times with anhydrous ethanol (5 min each), followed by rehydration in distilled water. Protease K working solution was applied to the tissue sections and incubated at 37°C for 22 min. The glass slides were then placed in PBS (pH 7.4) and washed three times on a shaker (5 min each). After drying, the tissue sections were covered with a membrane-breaking working solution and incubated at 37°C for 20 min. The slides were rinsed in PBS (pH 7.4) three times (5 min each), and dried again.

The tissues were treated with a mixture of TDT enzyme, dUTP, and buffer (volume ratio: 1:5:50) at 37°C for 10 min. The slides were placed flat in a humidified chamber with a small amount of water to maintain humidity and incubated at 37°C for 1 h. After incubation, the slides were washed three times with PBS (pH 7.4; 5 min each).

DAPI (4',6-diamidino-2-phenylindole) staining solution was applied to the tissue sections and incubated at 37°C for 10 min. After rinsing and drying, an anti-fluorescence quenching sealing agent was added. The tissue sections were observed under a fluorescence microscope, and images were captured. For DAPI-labeled nuclei, the excitation wavelength was set at 330–380 nm, and the emission wavelength was set at 420 nm. To observe FITC (Fluorescein Isothiocyanate) fluorescein-labeled apoptotic nuclei, the excitation wavelength was set at 465–495 nm, and the emission wavelength was set at 515–555 nm.

Determination of cortisol in serum

The cortisol level in serum was determined using a cortisol ELISA kit (H094-1-1, MALLBIO, Jiangsu, China). Serum samples were diluted 10-fold with the provided extraction solution, and subsequent steps were performed according to the manufacturer's instructions.

Determination of lactate

The lactate level in serum and muscle was determined using a lactate assay kit (A019-2-1, Jiancheng, Jiangsu, China). For muscle samples, frozen pork (100 mg) was homogenized in 0.9 mL of physiological saline. The homogenate was centrifuged at 4°C and $3,000 \times g$ for 10 min, and the supernatant was collected. For serum samples, the serum was diluted 10-fold with physiological saline. Subsequent steps were performed according to the manufacturer's instructions.

Determination of glycogen

The glycogen content in muscles was determined using a Micro Glycogen Assay Kit (KTB1340, Abbkine, Hubei, China). A 100 mg

frozen pork sample was mixed with 0.75 mL of extraction buffer and boiled for 20 min. After boiling, the samples were centrifuged at 25 °C and 8,000 × g for 10 min. The supernatant was collected and diluted to 5 mL with deionized water. Glycogen levels were measured according to the manufacturer's instructions.

Determination of ATP

The Enhanced ATP Assay Kit (S0027, Beyotime, Shanghai, China) was utilized to determine ATP. A frozen pork sample (100 mg) was homogenized in 1 mL of lysis solution, and the homogenate was centrifuged at 4 °C and 12,000 g for 5 min. The supernatant was collected, and the subsequent steps were performed according to the manufacturer's instructions.

Determination of MFI in pork at 24 h postmortem

A meat sample (2 g) was homogenized in 20 mL of MFI solution (containing 100 mmol/L KCl, 20 mmol/L K₂HPO₄/KH₂PO₄, 1 mmol/L EGTA, and 1 mmol/L MgCl₂) at 12,000 rpm for 90 s. The homogenate was centrifuged at 3,000 g for 15 min at 4 °C. The precipitate was resuspended in 20 mL of MFI solution and centrifuged again under the same conditions. The precipitate was collected and resuspended in 15 mL of MFI solution, followed by filtration through two layers of filter paper to obtain the myofibrillar protein solution. After adjusting the protein concentration of the solution to 0.5 mg/mL, the absorbance at 540 nm was measured immediately. The MFI was calculated as the absorbance multiplied by 200.

Determination of caspase-3 activity in pork

A commercial kit (BC3830, Solarbio, Beijing, China) was employed to determine the caspase-3 activity. Briefly, a meat sample (100 mg) was homogenized in 1 mL of reagent II (extraction solution), centrifuged at 4 °C and 15,000 g for 10 min, and the supernatant was collected and placed on ice for subsequent analysis. The caspase-3 activity was determined following the manufacturer's instructions.

Western blot analysis

A meat sample (100 mg) was homogenized in 1 mL of RIPA lysis solution (containing protease inhibitor cocktail and phosphatase inhibitor), ultrasonicated for 3 s, incubated for 30 min, and centrifuged at 15,000 g for 10 min. The pellet was resuspended in 0.4 mol/L KCl solution and centrifuged at 15,000 g for 20 min. The protein concentration of the supernatant was measured using the BCA Protein Assay Kit. The protein sample was mixed with loading buffer and heated in a metal bath at 95 °C for 10 min. Protein samples (10 µL) were loaded onto 4%–12% SDS-PAGE gels, and electrophoresis was performed at 60 V for 30 min followed by 120 V for 90 min. After electrophoresis, the proteins were transferred onto a nitrocellulose (NC) membrane. The membrane was blocked with 5% BSA in TBST for 1 h and then incubated with primary antibodies at 4 °C overnight. The membrane was washed five times with TBST

(containing 0.1% Tween-20), incubated with secondary antibodies for 2 h, and washed again five times with TBST. Protein bands were visualized using enhanced chemiluminescence with an Amersham ImageQuant 800 system, and band intensity was quantified using ImageJ software.

Statistical analysis

The effect of stunning methods on the measured variables was analyzed using one-way analysis of variance (ANOVA). Differences among groups were assessed using Duncan's multiple-range test. The significance level was set at $p < 0.05$. Data analyses were performed using SPSS (version 24, IBM, USA), and figures were prepared using Origin (version 2020, OriginLab, USA). All data are expressed as mean ± standard deviation (SD).

Results and discussion

Carbon dioxide stunning reduced stress

The levels of lactate and cortisol in the serum of pigs in the ES group were significantly higher than those in the CS group ($p < 0.001$; Fig. 1a & b). When animals encounter stress, the hypothalamic-pituitary-adrenal (HPA) axis is activated, leading to the release of corticotropin-releasing factor from the hypothalamus. The anterior pituitary gland releases corticotropin, which stimulates the adrenal cortex to produce and release glucocorticoids, thereby accelerating protein and fat metabolism to generate glucose^[8]. Therefore, electrically stunned pigs experience higher preslaughter stress levels. In addition, to confront or evade danger, animals often exhibit abnormal behaviors. Electrical shocks may induce excessive activity in the peripheral nervous system and muscle contractions throughout the body, while carbon dioxide stunning may trigger escape attempts and convulsions^[9]. Intense muscle twitching accelerates anaerobic glycolysis in muscles, leading to lactate production. Therefore, the higher stress levels in the electrical stunning group resulted in more intense preslaughter convulsions and higher serum lactate levels^[10,11]. These results are consistent with those of Hambrecht et al., who reported higher serum cortisol and lactate levels in electrically stunned pigs, indicating that electrical stunning induces greater stress and more severe seizures^[12]. However, numerous studies have also demonstrated the advantages of electrical stunning in reducing pre-slaughter stress^[13,14]. In general, equipment conditions and parameters significantly influence the stunning effect, which may explain the discrepancies observed across different studies.

Carbon dioxide stunning improved meat quality attributes

The pH of electrically stunned pork was significantly lower than that of the CS group at 1, 5, and 10 h postmortem ($p < 0.001$; Fig. 2a).

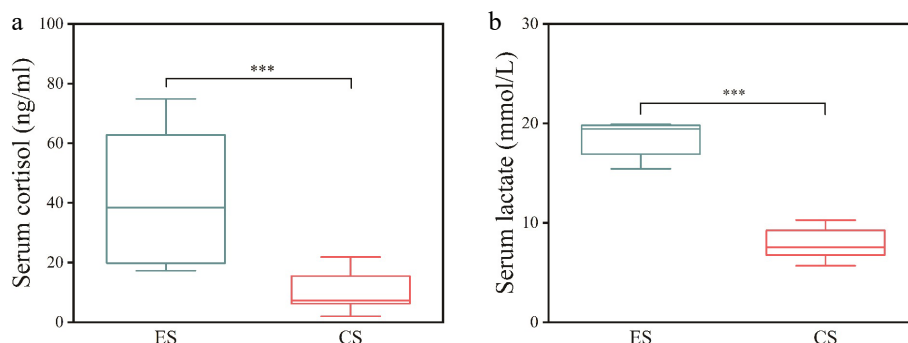


Fig. 1 Effect of stunning methods on preslaughter stress of pigs. (a) Cortisol content in serum, (b) lactate content in serum. ***, $p < 0.001$.

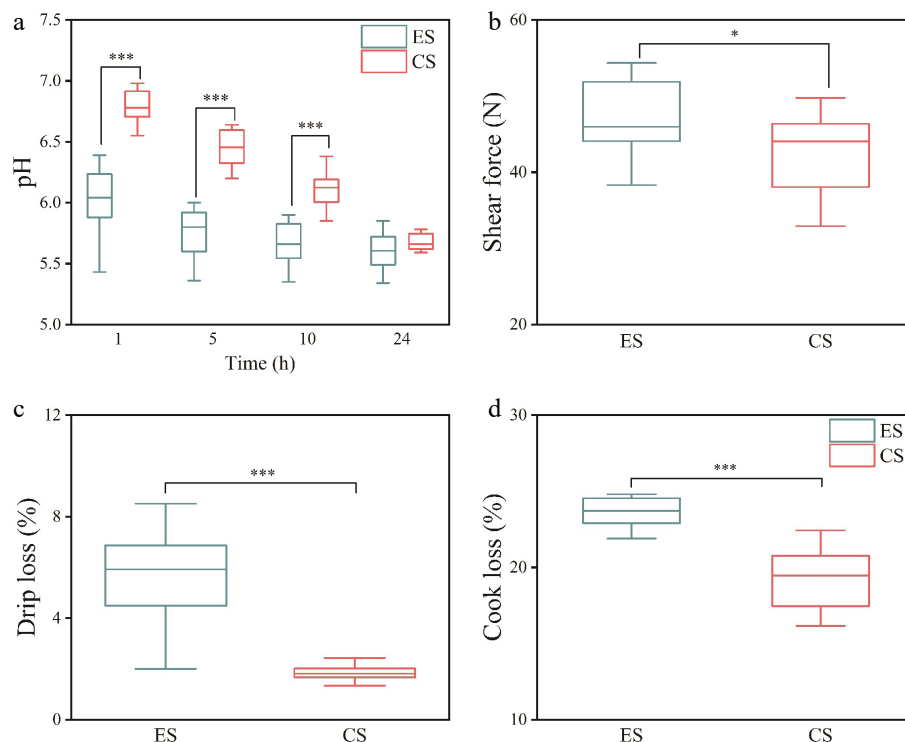


Fig. 2 Effect of stunning methods on pork quality. (a) pH, (b) shear force at 24 h, (c) drip loss, (d) cooking loss. *, $p < 0.05$, ***, $p < 0.001$.

Table 1. The effect of electrical stunning and carbon dioxide stunning on color in pork (mean $\pm$ SE).

Stunning method	Postmortem time (h)	L^*	a^*	b^*
Electrical stunning	1	45.46 $\pm$ 1.14 Ba	3.20 $\pm$ 0.66 Aa	2.12 $\pm$ 0.25 Ba
	5	47.56 $\pm$ 1.24Aab	3.97 $\pm$ 0.66 Aa	3.23 $\pm$ 0.26 Aa
	10	48.72 $\pm$ 1.16 Aa	4.09 $\pm$ 0.55 Aa	3.83 $\pm$ 0.23 Aa
	24	50.10 $\pm$ 0.72 Aa	4.73 $\pm$ 0.54 Aa	3.56 $\pm$ 0.27 Aa
Carbon dioxide stunning	1	44.41 $\pm$ 0.36 Ba	3.02 $\pm$ 0.38 Aa	2.02 $\pm$ 0.14 Ba
	5	43.44 $\pm$ 0.30 Bb	3.23 $\pm$ 0.38 Aa	2.04 $\pm$ 0.17 Ba
	10	44.68 $\pm$ 0.62 Bb	3.09 $\pm$ 0.50 Aa	2.90 $\pm$ 0.55 Ba
	24	47.75 $\pm$ 0.38 Ab	2.77 $\pm$ 0.35 Ab	3.92 $\pm$ 0.24 Aa

Different uppercase letters indicate significant differences in pork color at different postmortem times ($p < 0.05$), while different lowercase letters indicate significant differences in meat color due to different stunning methods ($p < 0.05$).

Pork from the CS group exhibited significantly better tenderness ($p < 0.05$; Fig. 2b). The L value of the ES group was significantly higher than that of the CS group at 10 and 24 h postmortem (Table 1). Electrical shocks induced severe tetanic contractions in the muscles of pigs, leading to the utilization of glycogen in skeletal muscle cells for anaerobic metabolism to generate energy. This resulted in a lower pH in the pork from the electrical stunning group during the early postmortem period. A rapid decline in muscle pH after slaughter can lead to protein denaturation and modification, significantly affecting meat quality attributes such as tenderness, water-holding capacity, and color. In normal muscle tissue, myofibrillar proteins are arranged in an ordered structure that efficiently scatters incident light. When pH declines rapidly, the myofibrillar proteins denature, losing their structural organization, while the sarcomere architecture becomes disrupted. This structural disordering reduces light-scattering efficiency, resulting in increased light absorption. Consequently, the meat surface exhibits a pale appearance^[15]. In skeletal muscles, water binds to charged proteins through adsorption. A decrease in pH alters the charge of proteins,

thereby affecting their ability to bind water. As the pH decreases and approaches the isoelectric point of the proteins, their ability to bind water diminishes, leading to water loss and reduced water-holding capacity in the meat. The decline in pH may induce denaturation of myofibrillar proteins and muscle contraction, thereby affecting pork tenderness.

Compared to carbon dioxide stunning, electrical stunning is associated with a higher incidence of pale, soft, and exudative (PSE) pork, as reflected in water-holding capacity (WHC), L value, and tenderness^[16].

Carbon dioxide stunning alleviated the muscle shortening at early postmortem time.

H&E staining revealed that the muscle fibers in the ES group were more densely arranged at 1 h postmortem (Fig. 3a). Quantitative analysis of intercellular spaces indicated that the muscle intercellular space in carbon dioxide-stunned pork was significantly larger than that in electrically stunned pork at 1 and 24 h ($p < 0.01$; Fig. 3b). The myofibrillar fragmentation index (MFI) of pork samples in the CS group was significantly higher than that in the ES group at 24 h postmortem ($p < 0.05$; Fig. 3c). The size of muscle fibers is closely related to meat tenderness, with finer muscle fibers generally associated with greater tenderness^[17]. The myofibrillar fragmentation index (MFI) has been demonstrated to positively correlate with meat tenderness. At 24 h postmortem, the higher MFI in the CS group indicated a greater degree of proteolysis compared to the ES group. This suggests that proteolytic enzyme activity differs between the two groups postmortem. The difference in intercellular space at 1 h postmortem was primarily due to muscle contraction during stunning, with tetanic contractions induced by electrical shocks resulting in densely arranged muscle fibers. After slaughter, calcium enters the cytoplasm due to disruption of the sarcoplasmic reticulum, activating the formation of actin-myosin cross-bridges and triggering contraction of the entire muscle fiber. When ATP levels fall below a critical threshold, actin-myosin cross-bridges cannot be

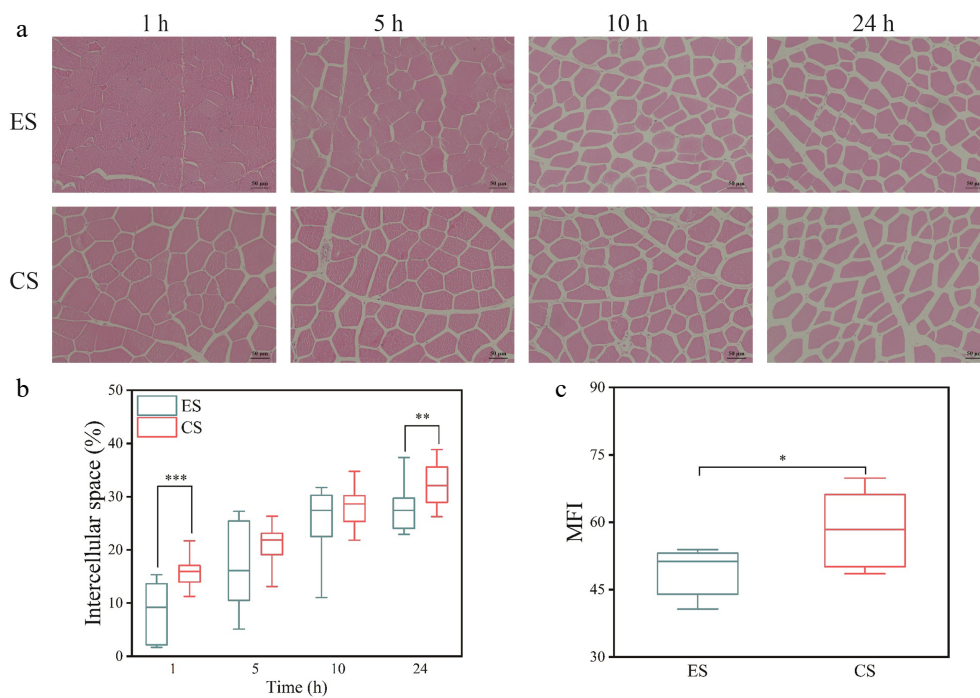


Fig. 3 Effect of stunning methods on muscle fiber structure. (a) HE staining images, (b) intercellular space of HE staining, (c) MFI at postmortem 24 h.

disengaged, leading to the onset of rigor mortis^[18]. The varying degrees of postmortem muscle contraction indicate differences in postmortem energy metabolism between the two groups of pigs. However, the muscle fiber structure at 24 h postmortem was minimally influenced by preslaughter stunning. The observed differences in protein degradation between the two groups at 24 h postmortem may be attributed to the sustained activity of proteases.

Electrical stunning significantly accelerated postmortem energy metabolism

At 1 h postmortem, the glycogen and ATP contents in the CS group were significantly higher than those in the ES group, whereas the lactate content was significantly lower ($p < 0.01$; Fig. 4a–c). At 5 and 10 h postmortem, the lactate content in the CS group muscles was significantly lower than that in the ES group ($p < 0.01$). At 24 h, no significant differences were observed in the contents of glycogen, ATP, or lactate ($p > 0.05$).

Preslaughter stunning likely accounts for the observed differences in meat quality attributes. During the production of chilled meat, muscles are subjected to a harsh environment characterized by low temperature, hypoxia, and energy depletion following slaughter. The residual glycogen in skeletal muscle cells is metabolized through anaerobic glycolysis^[19,20]. Severe convulsions induced

by electrical shocks resulted in the rapid depletion of glycogen and ATP in the muscles, the accumulation of lactic acid, and an accelerated rate of anaerobic glycolysis postmortem. This study demonstrated that lactate levels in the muscles of electrically stunned pigs were elevated, whereas glycogen and ATP levels were reduced^[21]. These changes can alter the degree of protein-water binding, cause cellular moisture loss, modify the ratio of oxygenated to deoxygenated myoglobin, and ultimately impact color, tenderness, and water-holding capacity (WHC).

Carbon dioxide stunning induced cell apoptosis.

During the conversion of skeletal muscle into meat, cellular apoptosis plays a pivotal role. Caspases, activated during apoptosis, not only directly participate in the degradation of myofibrillar proteins but also facilitate the activation of calpains by degrading calpastatin, a specific inhibitor of calpains. TUNEL staining results revealed a gradual increase in apoptotic cells in the CS group over storage time, whereas the ES group exhibited no apoptosis (Fig. 5a). Caspase-3 activity was significantly higher in the CS group at 1 and 5 h postmortem (Fig. 5b), indicating a higher incidence of apoptosis in this group. Necroptosis, an alternative pathway to apoptosis under energy-deficient conditions, showed higher MLKL phosphorylation and incidence in the ES group at the same time points (Fig. 5b).

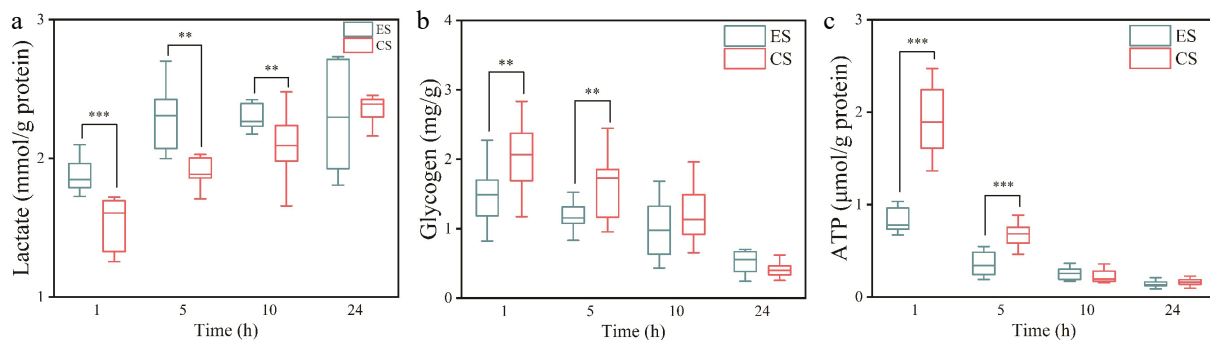


Fig. 4 Effect of stunning methods on postmortem energy metabolism of skeletal muscle cells. (a) Lactate, (b) glycogen, (c) ATP. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

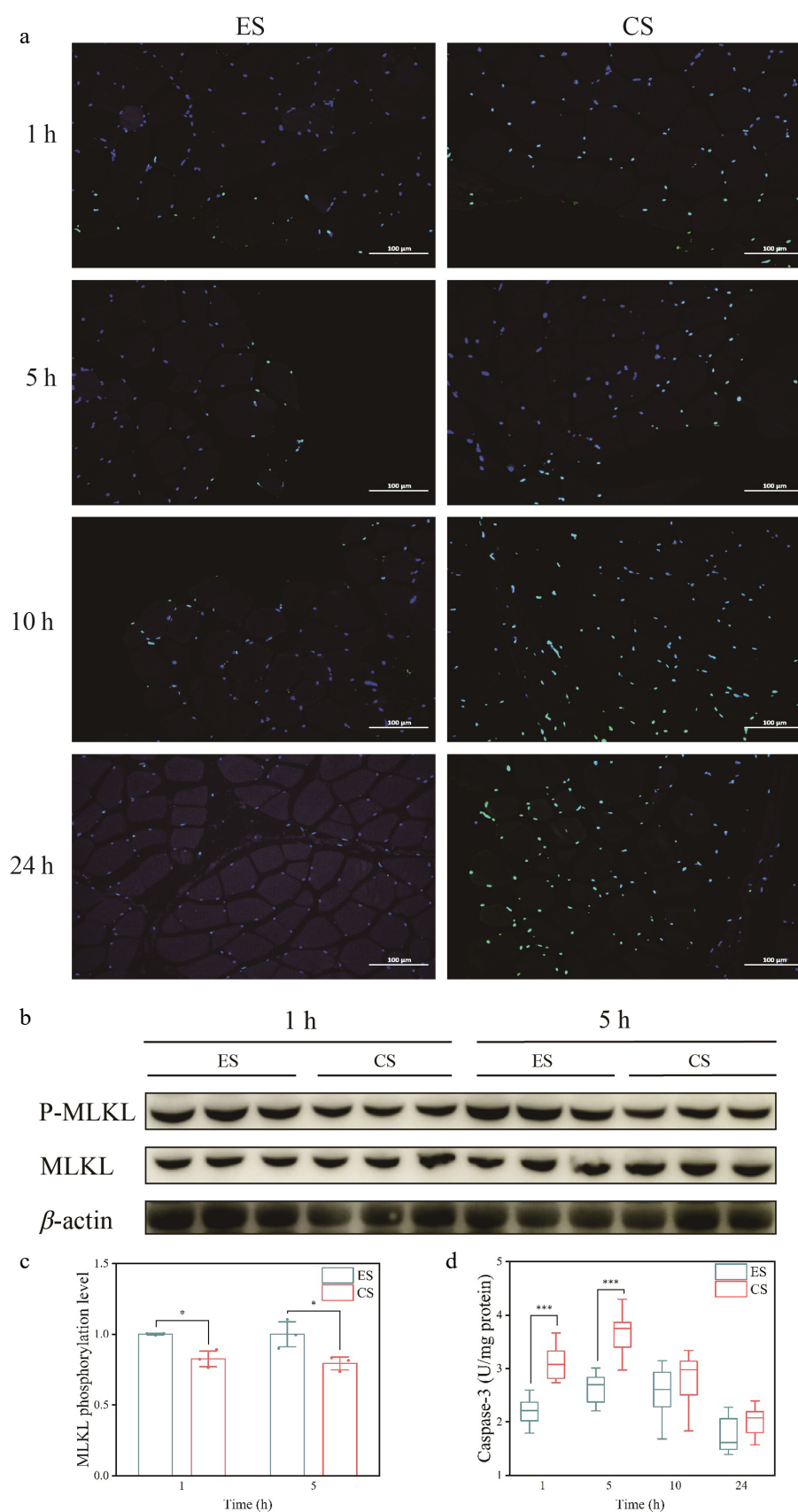


Fig. 5 Effect of stunning method on cell death. (a) TUNEL staining images, (b) Western blotting images of MLKL and phosphor-MLKL, (c) MLKL phosphorylation level, (d) caspase-3 activity. *, $p < 0.05$, ***, $p < 0.001$.

μ -Calpain is known to degrade proteins such as nebulin, titin, troponin T, and desmin, disrupting the muscle's ultrastructure, particularly weakening and destroying the Z-line, thereby

promoting myofibril fragmentation and meat tenderization^[22]. However, μ -calpain alone cannot fully account for protein degradation and tenderness changes, explaining only about 30% of

tenderness variations post-slaughter. Even in the presence of Ca^{2+} chelators and calpain inhibitors, some myofibrillar proteins still degrade, suggesting the involvement of caspases. Studies have shown that caspase activity peaks early in postmortem tenderization, indicating it may be the first endogenous enzyme active in muscle. Caspases are implicated in myofibrillar protein degradation and play a significant role in meat tenderization, potentially interacting with calpains to enhance tenderness^[23]. The activation pathways of these proteases are precisely regulated by cell death modes, making cell death mode, targeting a potential novel strategy for controlling meat tenderness.

Intracellular energy levels are crucial in determining cell death mode. Sufficient ATP induces pyroptosis, parthanatos, autophagy-dependent cell death, oxeiptosis, and apoptosis, whereas necroptosis and alkaliphotosis are less ATP-dependent. ATP-dependent cell death modes activate protease systems, with pyroptosis and apoptosis activating caspases and autophagy activating the proteasome and cathepsin^[24–27]. The transition from apoptosis to necrosis can be induced by depleting intracellular ATP under the same stimuli^[28]. Thus, ATP levels in muscle cells determine cell death mode, influence protease activity, and ultimately regulate meat quality.

Pre-slaughter stunning significantly affects the postmortem skeletal muscle cell microenvironment, with improper stunning potentially depleting glycogen and ATP and accumulating lactic acid, further impacting postmortem muscle energy levels and pH.

Postmortem muscles exhibit a coexistence of various cell death modes, with apoptosis, autophagy, and necrosis being the endpoints of skeletal muscle cells^[29]. ATP, a vital energy source in skeletal muscle cells, is maintained post-slaughter through biochemical pathways like ion channel regulation, signal transduction, and gene transcription^[30]. ATP content dictates skeletal muscle cell death manner, with mild depletion leading to apoptosis and severe depletion to necrosis. Apoptosis, an energy-consuming process requiring new protein synthesis, contrasts with necrosis, an ATP-independent passive process^[31]. Post-slaughter, muscles face a harsh environment of hypoxia, low temperature, and energy deprivation, making apoptosis the preferred mode for maintaining homeostasis. However, pre-slaughter glycogen and ATP depletion in the ES group may reduce the number of cells capable of undergoing apoptosis compared to the CS group, leading to a higher incidence of necrosis in the ES group due to ATP depletion during apoptosis^[32].

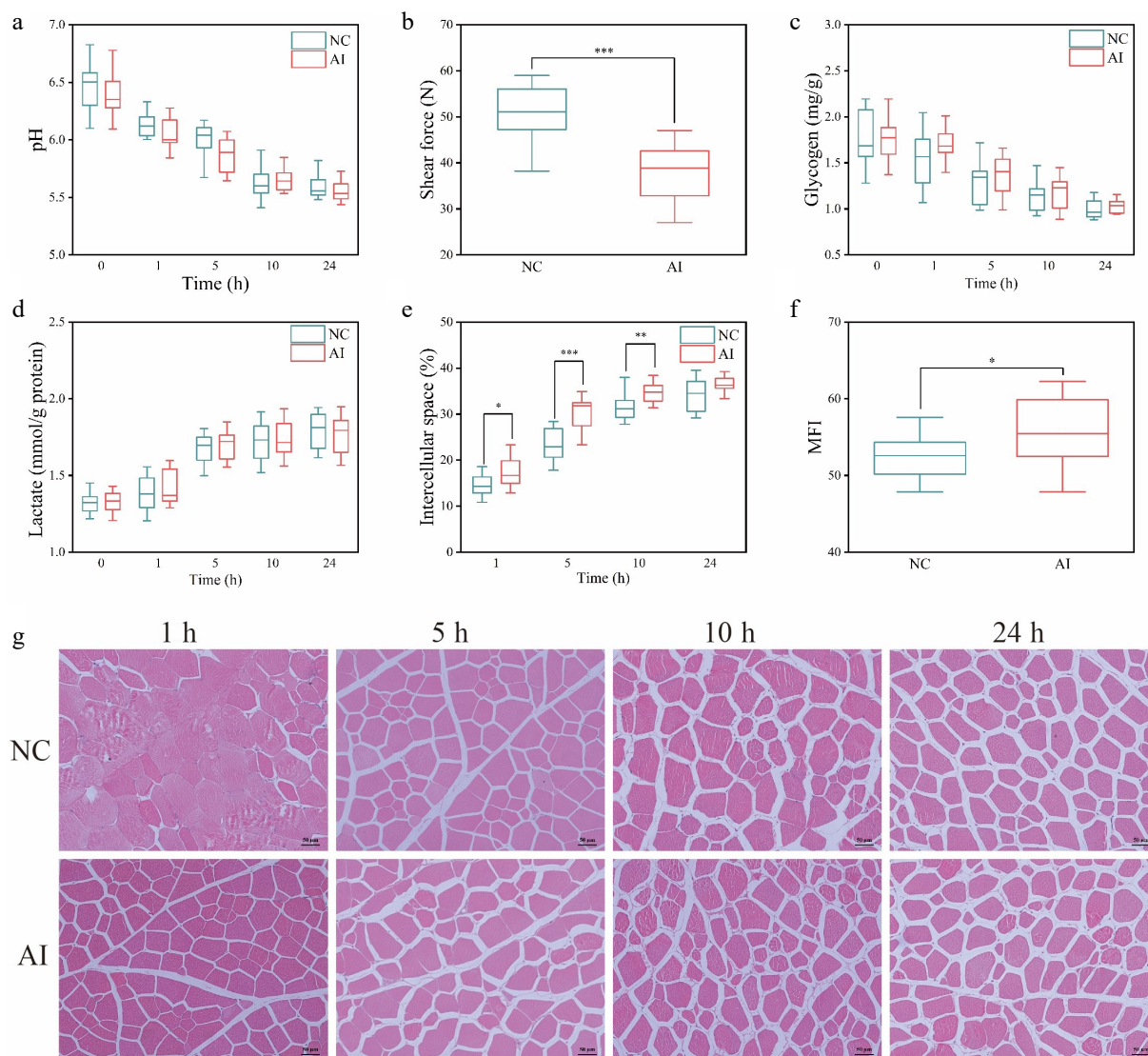


Fig. 6 Effect of ATP treatment on the meat quality of electrically stunned pork. (a) pH, (b) shear force at 24 h, (c) glycogen content, (d) lactate content, (e) intercellular space of HE staining, (f) MFI at 24 h, (g) HE staining. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

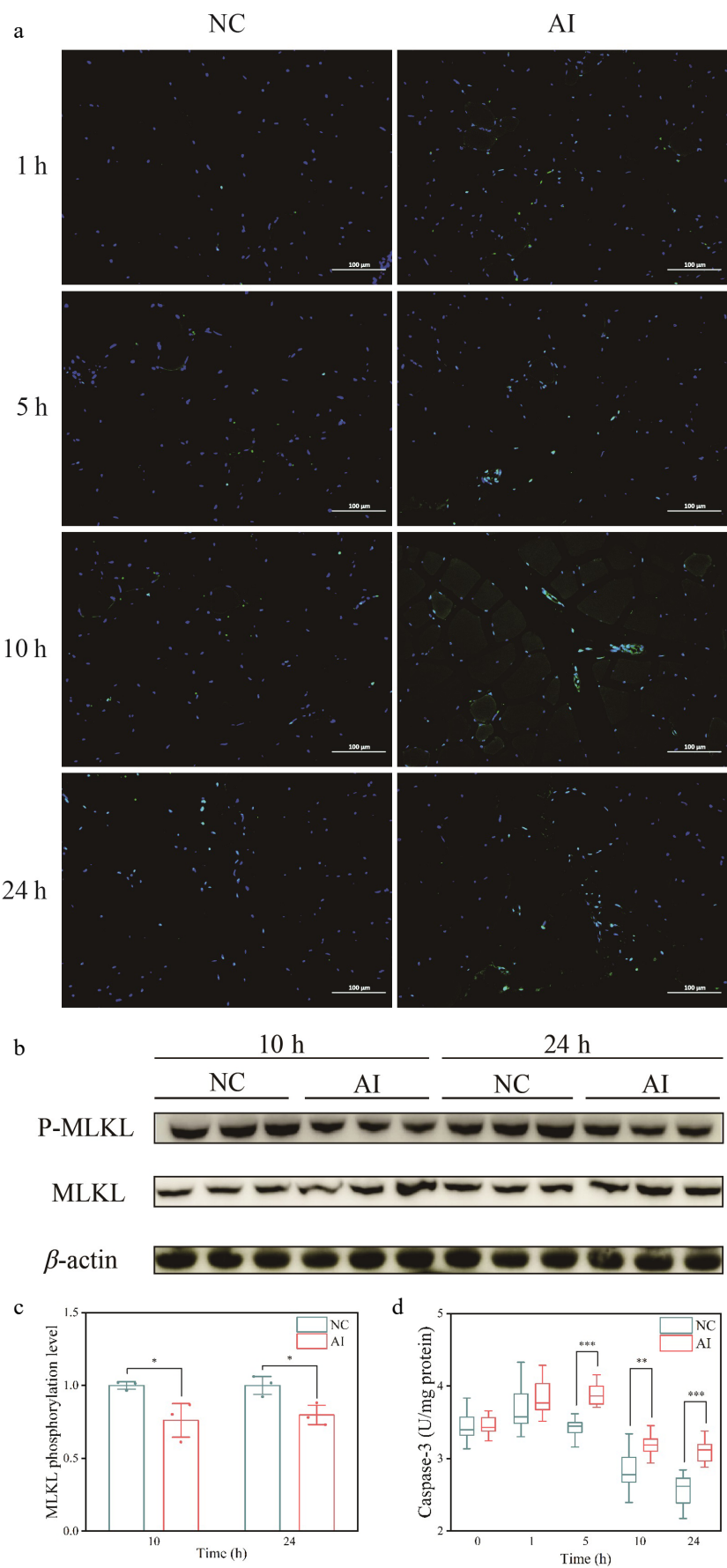


Fig. 7 Effect of ATP treatment on skeletal muscle cell death. (a) TUNEL staining images, (b) Western blotting image of MLKL and phosphor-MLKL, (c) MLKL phosphorylation level, (d) caspase-3 activity. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

Endogenous proteases, particularly μ -calpain, and caspases, play crucial roles in postmortem meat tenderization. μ -Calpain degrades myofibrillar proteins, improving tenderness, while caspases also significantly contribute to tenderization^[33,34]. Caspase-3 enhances tenderness through the calpain/calpastatin system, with calpain affecting tenderization by regulating apoptosis and myofibrillar protein degradation^[35]. Thus, calpain-induced apoptosis has a more significant impact on postmortem meat tenderization than its direct effect on protein degradation.

This study highlights significant differences in skeletal muscle cell death modes under different pre-slaughter stunning conditions, likely due to varying cellular environments and energy levels. Electrical stunning, leading to lower pH and energy levels in postmortem muscle, may induce a shift from apoptosis to necrosis^[36,37]. A higher apoptosis rate activates more caspases, contributing to postmortem pork tenderization.

ATP supplementation improved the meat quality of electrically stunned pigs

ATP may influence the mode of death of postmortem skeletal muscle cells and subsequently affect meat quality. To investigate this, muscles from electrically stunned pigs were supplemented with ATP.

As illustrated in Fig. 6a–d and Table 2, ATP treatment significantly enhanced the tenderness of pork from electrically stunned pigs ($p < 0.001$), but had minimal impact on meat color, pH, glycogen, and lactate content. ATP treatment increased the muscle fiber space at 1, 5, and 10 h ($p < 0.01$), with a significant rise in MFI at 24 h postmortem. This suggests that higher ATP levels in postmortem muscles are associated with increased protein degradation. TUNEL staining (Fig. 7a), or caspase-3 activity (Fig. 7c) in postmortem pork. This could be attributed to ATP treatment increasing the apoptosis rate of skeletal muscle cells by reducing MLKL phosphorylation (Fig. 7b), thereby further promoting necroptosis.

Glycolysis is a critical process that generates ATP to meet energy demands and produces H^+ , which lowers pH and influences meat color, tenderness, and water-holding capacity (WHC)^[38]. Bai et al. have highlighted that ATP serves as a key substrate for protein phosphorylation^[39]. In this study, the degree of myofibrillar protein phosphorylation in the ATP-treated group was significantly higher than in the control group, indicating that ATP affects meat quality, at least in part, through protein phosphorylation.

Research has shown that carbon dioxide stunning, compared to electrical stunning, results in less stress, lower lactate dehydrogenase (LDH) activity, higher ATP content, and superior meat quality, making it a more recommended stunning method^[40]. Additionally,

creatine monohydrate can elevate phosphocreatine levels in muscles, thereby increasing ATP levels through phosphocreatine degradation. A study found that supplementing chicken diets with 1,200 mg/kg of creatine monohydrate two weeks before slaughter effectively reduced pre-slaughter stress and improved meat quality^[41].

Conclusions

Compared to electrical stunning, CO_2 stunning improved the WHC, tenderness, and pH of pork while reducing the L value. The postmortem death mode of skeletal muscle cells represents a potential pathway through which different pre-slaughter stunning methods influence pork quality. This study demonstrates that the postmortem ATP content in animal muscles is a critical factor in determining final meat quality. Different pre-slaughter stunning methods induce varying stress responses in pigs, which subsequently affect glycolysis in skeletal muscle cells, leading to differences in ATP and lactate levels. These variations in energy and pH levels can regulate distinct modes of cell death in skeletal muscle cells. Higher ATP levels tend to favor apoptosis, activating caspases to promote tenderization, whereas ATP depletion may shift the cell death mode toward necrosis or necroptosis. However, the specific effects of electric shock and hypoxia-induced suffocation on meat quality attributes require further investigation.

Authors contributions

The authors confirm their contributions to the paper as follows: writing – original draft preparation, methodology, data curation: Qiu X; data acquisition: Qiu X, Mao X; data analysis: Qiu X, Mao X, Shan K; supervision, writing – review & editing: Shan K, Li C; conceptualization, resources, project administration, data confirmation, experimental design, experiment materials: Li C. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The datasets generated during and/or analyzed in the current study are available from the corresponding author on reasonable request.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Table 2. Effect of ATP treatment on color in electrically stunned pork (mean $\pm$ SE).

Injection	Postmortem time (h)	L^*	a^*	b^*
NS	0	38.02 $\pm$ 0.38Ca	6.48 $\pm$ 0.22ABa	1.07 $\pm$ 0.08Ca
	1	37.79 $\pm$ 0.53Ca	6.08 $\pm$ 0.39ABa	1.36 $\pm$ 0.06Ca
	5	40.34 $\pm$ 0.91Ba	6.98 $\pm$ 0.30Aa	2.19 $\pm$ 0.19Ba
	10	44.79 $\pm$ 0.67Aa	6.01 $\pm$ 0.30Ba	2.12 $\pm$ 0.10Bb
	24	45.39 $\pm$ 0.78Aa	6.73 $\pm$ 0.28ABa	2.89 $\pm$ 0.21Ab
ATP	0	38.68 $\pm$ 0.35Ba	6.33 $\pm$ 0.21ABa	1.17 $\pm$ 0.06Ca
	1	39.37 $\pm$ 0.69Ba	6.23 $\pm$ 0.23ABa	1.47 $\pm$ 0.08Ca
	5	40.67 $\pm$ 0.75Ba	7.26 $\pm$ 0.59Aa	2.55 $\pm$ 0.10Ba
	10	43.49 $\pm$ 0.83Aa	5.48 $\pm$ 0.35Ba	2.75 $\pm$ 0.15Ba
	24	44.66 $\pm$ 0.85Aa	5.94 $\pm$ 0.37Ba	3.53 $\pm$ 0.11Aa

Different uppercase letters indicate significant differences in pork color at different postmortem times ($p < 0.05$), while different lowercase letters indicate significant differences in meat color due to ATP treatment ($p < 0.05$).

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