The effect of postmortem pH decline rate on mitochondrial apoptosis and tenderness of bovine skeletal muscle during aging

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Abstract

This study aimed to investigate the effect of postmortem pH decline rate on mitochondrial dysfunction-induced apoptosis and bovine muscle tenderness during aging. Protein denaturation, reactive oxygen species (ROS) levels, mitochondrial apoptosis factors, and shear force were assessed in bovine muscles with different pH decline rates. The results showed that compared to the slow group, fast pH decline in bovine muscle significantly increased sarcoplasmic protein denaturation at 6–12 h and mitochondrial ROS levels at 6–24 h ($P<0.05$), and in turn significantly accentuated mitochondrial dysfunction and cytochrome c oxidation levels ($P<0.05$), resulting in caspase-3 activation, apoptosis, and reduced muscle shear force ($P<0.05$). These results demonstrated that the fast postmortem pH decline enhanced mitochondrial apoptosis and bovine muscle tenderization by inducing ROS accumulation during aging.

Introduction

Tenderness is one of the most important factors that influence consumer satisfaction with meat and contribute to repeat purchases^{1,2}. After slaughter, the occurring chemical and physical changes contribute to the development of muscle into the meat and promote muscle tenderization³. Among these factors, glycolysis is one of the most essential processes in the production of final meat quality, and the rate of glycolysis in the early postmortem period may have a significant impact on muscle tenderization progression⁴. The inconsistency in meat tenderness could be attributed to variations in the rate of pH reduction in the early postmortem period^{5,6}. Previous reports have suggested that the bovine muscle with a low pH before the onset of rigor mortis was transformed into tender beef^{7,8}. These results suggest that the rate of postmortem pH decline had an important influence on muscle tenderization.

Muscle tenderization is influenced by the extensive interactions of biochemical processes in carcasses during postmortem aging, which includes the hydrolysis of key myofibrillar proteins such as desmin, titin, and troponin-T⁹. The hydrolysis of myofibrillar protein is the result of the activation of endogenous proteases, such as calpains, cathepsins, and caspases^{10,11}. For decades, mitochondrial apoptosis has been shown to play an important part in the breakdown of myofibrillar protein in muscle throughout aging^{12,13}. In the mitochondrial apoptosis pathway, release of cytochrome c from mitochondria provided the stimulus for caspase-9 activation through the association of apoptotic protein activator-1 (Apaf-1) and procaspase-9 in the cytoplasm, and subsequently activated caspase-9 followed by caspase-3 activation thus enhancing muscle tenderness¹⁴. This type of cell death was different from necrosis, it was a process of programmed death caused by a lack of intracellular energy¹⁵. Furthermore, previous research has demonstrated that cytoplasmic acidification caused apoptosis and caspase-3 activation, suggesting that cytoplasmic pH changes occur early in the induction of mitochondrial apoptosis-related pathways¹⁶. After slaughter and bleeding of the cattle, the oxygen supply to the muscle is disrupted, and the aerobic respiration in the cells is switched to anaerobic glycolysis, resulting in lactate accumulation, ATP hydrolysis, and subsequently muscle acidification¹⁷. During postmortem aging, a fall in pH may further promote mitochondrial dysfunction and lead to apoptosis in bovine muscle^{18,4}. In a low pH milieu, Reshkin et al.¹⁹ discovered that various apoptosis-related enzymes, namely DNases and caspases, were activated. As a result, acidification causes mitochondrial apoptosis in muscle cells. However, there is still a lack of conclusive evidence connecting pH decline rate to mitochondrial apoptosis in postmortem muscle.

Thus, in the present work, the bovine carcasses of various pH in the early postmortem phase were selected to investigate the influence of pH decline rate on mitochondrial apoptosis and muscle tenderness during aging. We aimed to clarify the mechanism of postmortem pH regulating muscle tenderness from the mitochondrial apoptosis pathway by analyzing protein denaturation temperature, ROS levels, mitochondrial apoptosis factors, myofibrillar fragmentation index (MFI), and shear force in different pH decline rates groups of bovine muscles.

Results And Discussion

Changes in pH of bovine muscle during postmortem aging. The pH of bovine muscle with different pH decline rates during postmortem aging is shown in Fig. 1. The pH of the fast pH decline group decreased significantly at 24 h while the pH of the slow group decreased significantly at 48 h compared with 0.5 h ($P<0.05$). However, the pH in the fast group was lower than the slow group by 4.06%, 5.11%, 8.34%, 4.43%, and 2.67% at 0.5, 2, 6, 12, and 24 h ($P<0.05$), respectively. These findings suggest a significant difference in pH decline rates between the two groups within 24 h postmortem.

After slaughter and bleeding, glycogen metabolism shifts from aerobic to anaerobic and the metabolite lactate accumulates, resulting in a decline in muscle pH^{24,25}. Apaoblaza et al.²⁶ demonstrated that the ultimate pH of carcasses was correlated with glycogen content and a sufficient amount of glycogen can supply the necessary fuel for the pH decrease. Previous studies have shown that the breakdown of ATP produced by postmortem muscle glycolysis led to the accumulation of protons (H^+), which was also caused a decrease in pH²⁷. The rate of glycolysis is determined by the activity of glycolytic enzymes. Pyruvate kinase, enolase 2, phosphoglucosmutase 1, fructose diphosphate aldolase, and enolase 3 are all phosphorylation-regulated enzymes that may be implicated in postmortem muscle glycolysis²⁸. In addition, several other pre- and post-mortem factors might affect postmortem muscle glycolysis, such as genetics, pre-slaughter stress, carcass temperature, and S-nitrosylation of glycolytic enzymes, etc.^{26,29,30}. In the present study, the fast group of muscles may have consumed glycogen more quickly, resulting in a low pH in the early postmortem period.

Effect of pH decline rate on protein denaturation and mitochondrial ROS accumulation in postmortem bovine muscle. The decrease in pH is generally thought to induce further protein denaturation in muscle. Therefore, we evaluated the influence of pH decline rate on muscle protein denaturation temperature and ROS levels. A total of three heat absorption peaks were observed in the muscle tissue by DSC scanning, representing the denaturation temperature of the myosin head, sarcoplasmic protein, and actin³¹. The denaturation temperature of a protein increases when its structure is slightly altered. As shown in Table 1, both myosin and sarcoplasmic protein denaturation temperatures increased significantly in both groups during postmortem aging ($P<0.05$), however myosin denaturation temperature in the fast group was 2.60% higher than in the slow group at 12 h ($P<0.05$). Sarcoplasmic protein denaturation temperature was 1.79% and 1.39% higher than in the slow group at 6 and 12 h, respectively ($P<0.05$). However, actin did not show any differences in denaturation temperature during the postmortem aging in the two groups. In addition, the fluorescence images of ROS in two groups are shown in Fig. 2A. The average fluorescence values of ROS in muscle cells, reflecting the ROS levels, were significantly higher in the fast group than the slow group at 6–24 h postmortem ($P<0.05$; Fig. 2B), with a most significant difference at 12 h ($P<0.01$). The above results suggested that fast pH decline promoted myosin and sarcoplasmic protein denaturation and ROS accumulation in the postmortem muscle.

Insufficient ATP production and supply in the postmortem muscle in conjunction with the reduction of pH disrupt the normal redox system, leading to the accumulation of proapoptotic free radicals such as ROS^{13,14}. There is a direct correlation between increased ROS levels and lower muscle pH³². Previous studies have shown that the antioxidant enzymes such as SOD, CAT, and GSH-Px showed decreased activity at the low pH in postmortem bovine muscle³³. Sun et al.³⁴ showed that an ultimate pH of 5.3–5.6 during postmortem muscle aging did not indicate that muscle glycogen was depleted, but rather that the inactivation of enzymes in the glycolytic pathway caused the reaction to halt when the pH fell below a certain threshold. These findings suggest that lower muscle pH induces a decrease in enzyme activity in the cytoplasm. The enzymes in the cytoplasm generally play an essential role in a neutral environment. Lower pH caused the active core group of the enzyme to deviate from its optimal dissociation state, reducing its capacity to bind to the substrate and catalytic activity^{25,35}. In the present study, the fast pH decline group had greater ROS levels than the slow group, which could be owing to changes in the structure and activity of ROS scavenging enzymes by rapidly declining muscle pH. Taken together, it was clear that postmortem fast pH decline contributed to the accumulation of muscle ROS by inducing sarcoplasmic protein denaturation.

Table 1

Changes in the denaturation peak temperature of Myosin (T_{Myosin}), Myoplasmic protein ($T_{\text{Myoplasmic protein}}$) and Actin (T_{Actin}) in bovine muscles of different glycolytic rates at 0.5, 6, 12, 24, 72, 120, and 168 h postmortem.

Indicators	Glycolytic rate	Postmortem aging time						
		0.5 h	6 h	12 h	24 h	72 h	120 h	168 h
T_{Myosin} (°C)	Fast	51.85±1.23 ^B	51.81±1.78 ^B	52.91±1.54 ^{AB*}	52.93±1.51 ^{AB}	53.18±1.39 ^{AB}	53.68±1.45 ^A	53.67±1.39 ^A
	Slow	51.73±1.34 ^b	51.63±1.36 ^b	51.53±1.43 ^b	52.89±1.29 ^{ab}	53.62±1.56 ^a	53.81±1.48 ^a	53.45±1.35 ^a
$T_{\text{Myoplasmic protein}}$ (°C)	Fast	61.38±1.44 ^B	62.64±1.73 ^{AB*}	63.21±1.33 ^{A*}	63.64±1.39 ^A	63.51±1.26 ^A	63.38±1.08 ^A	63.33±1.36 ^A
	Slow	61.46±1.36 ^c	61.54±1.47 ^c	62.35±1.36 ^b	63.39±1.28 ^a	63.24±1.04 ^a	63.61±1.27 ^a	63.40±1.63 ^a
T_{Actin} (°C)	Fast	76.43±0.75 ^A	76.61±0.58 ^A	76.31±0.57 ^A	77.53±0.61 ^A	77.47±0.65 ^A	76.88±0.59 ^A	76.59±0.78 ^A
	Slow	76.51±0.66 ^a	77.03±0.65 ^a	76.53±0.65 ^a	77.56±0.72 ^a	77.18±0.98 ^a	77.67±0.54 ^a	76.42±0.68 ^a

A-B: Each letter denotes a statistically significant difference in the fast group at different postmortem times ($P<0.05$). a-c: Each letter denotes a statistically significant difference in the slow group at different postmortem times ($P<0.05$). * indicates the significance compared the same time points between two groups ($P<0.05$). All measurements were expressed as the mean ± SE.

Effect of pH decline rate on mitochondrial dysfunction and cytochrome c redox state. A mitochondrial dysfunction is a precursor to cell apoptosis. Therefore, the effect of pH decline rate on mitochondrial membrane permeability and cytochrome c redox state were assessed. Fig. 3A showed that the mitochondrial membrane permeability significantly increased during postmortem aging in both groups ($P<0.05$) and significantly higher in the fast group by 14.05%, 22.39%, 18.34% and 25.28% than in the slow group at 6, 12, 24 and 72 h, respectively ($P<0.05$). The cytochrome c reduction levels are shown in Fig. 3B. Our results showed that cytochrome c reduction levels decreased significantly postmortem in both two groups ($P<0.05$). Furthermore, the cytochrome c reduction in the fast group was significantly lower than the slow group by 16.71%, 23.39%, 17.05%, and 26.61% at 6, 12, 24, and 120 h, respectively ($P<0.05$), indicating that fast pH decline induced mitochondrial membrane dysfunction and cytochrome c oxidation during the postmortem aging.

ROS, produced in mitochondria, was an additional indicator for mitochondrial apoptosis³⁶. The weakening of antioxidant enzymes and other defense systems in postmortem muscle inevitably led to the production of ROS³⁷. Excessive accumulation of ROS induced swelling and destruction of mitochondria membrane and ultimately mediated mitochondrial apoptosis^{14,38}. ROS could attack the mitochondrial membrane polyunsaturated fatty acids to produce lipid peroxides such as malondialdehyde, causing mitochondrial swelling and increasing mitochondrial membrane

permeability^{13,39}. Moreover, ROS induced cytoplasmic Ca^{2+} influx into mitochondria, leading to mitochondrial Ca^{2+} overload, which further caused an increase in mitochondrial membrane pore opening and permeability⁴⁰. Subsequently, the release of cytochrome c from damaged mitochondria into the cytoplasm was a universal event for mitochondrial apoptosis⁴¹, but its catalytic activity was largely dependent on its oxidative state¹⁴. Suto et al.⁴² also showed that the oxidized cytochrome c was more effective in initiating mitochondrial apoptosis. In the present study, fast pH decline facilitated cytochrome c oxidation. Previous studies have showed that the oxidation of cytochrome c was associated with mitochondrial oxidants, such as H_2O_2 ^{37,43}. Together with the above findings on the role of pH decline rate in ROS, it suggested that fast pH decline indirectly induced mitochondrial dysfunction and cytochrome c oxidation by promoting the accumulation of mitochondrial ROS.

Effect of pH decline rate on caspase-3, apoptosis and tenderness in bovine muscle. To further exam the downstream events of cytochrome c oxidation and mitochondrial dysfunction, the effect of pH decline rate on caspase-3 activity, apoptotic nucleus, MFI, and shear force were evaluated during postmortem aging. As depicted in Fig. 4, in both two groups, caspase-3 activity tended to increase initially and later decrease ($P<0.05$), with caspase-3 activity reaching the peak at 24 h. Besides, the caspase-3 activity in the fast group was significantly higher than the slow group at 12–168 h ($P<0.05$), except the 72 h time point. TUNEL assay of the apoptotic nucleus is shown in Fig. 5A. The normal nucleus was labeled with overall blue fluorescence (DAPI), while the apoptotic nucleus was labeled with green fluorescence (TUNEL positive). The proportion of apoptotic nuclei of the total nuclei was quantified and shown in Fig. 5B, with a significant increase in the total number of apoptotic nuclei in both groups ($P<0.05$). In the fast group, the total apoptotic nuclei were significantly higher than those in the slow group at 24–168 h ($P<0.05$).

The number of the MFI reflects the degree of fragmentation of myofibrils and positively correlates with the degradation of myofibrillar proteins. Table 2 showed a significant increase in MFI during aging for both groups ($P<0.05$), however, the fast group had a significantly higher MFI than the slow group at 12–168 h ($P<0.05$). Shear force was used to evaluate the tenderness of bovine muscle, and the smaller the value indicates the tenderer of the muscle. As shown in Table 2, the shear force of both fast and slow groups kept growing until 12 h ($P<0.05$). However, it plummeted at 24–168 h ($P<0.05$). In the fast group, the shear force was significantly higher than in the slow group at 6 h ($P<0.05$), but significantly lower than in the slow group at 12–168 h ($P<0.05$) and 10.49% lower than in the slow group at 168 h. The above results suggested that fast pH decline promoted caspase-3 activation, apoptosis, and bovine muscle tenderization during postmortem aging.

Caspase-3 is one of the most important effector caspases in the apoptotic pathway and plays a critical role in muscle tenderization⁴⁴. The oxidized cytochrome c interacts rapidly with apoptotic protease activating factor (Apaf-1) and procaspase-9 in the presence of dATP to activate caspase-9, which further activates the downstream effector caspase-3^{12,13,41}. The results of this study indicated that the caspase-3 showed the highest activity at 24 h time point. Our finding was confirmed by previous reports⁴⁵, in which they suggested that caspase-3 activity in bovine skeletal muscle gradually increased to a maximum at approximately 24 h time point. Besides, our study also showed that the caspase-3 activity in the fast group was higher than that in the slow group. Combining the above effect of pH decline rate on ROS accumulation, mitochondrial dysfunction, and cytochrome c oxidation, we concluded that the fast pH decline promoted postmortem caspase-3 activation and apoptosis in the bovine muscle.

Postmortem muscle tenderization is a complex biochemical process involving the degradation of myofibrillar proteins and is controlled by a variety of protein hydrolases, including caspase-3 and calpain-1⁴⁶. According to Kemp and Parr⁴⁷, the formation of 28- and 30-kDa troponin-T degradation products and the degradation of desmin improved the muscle tenderness. Although these proteins were more likely to be recognized by calpain-1, caspase-3 also played an important role in this process. Huang et al.⁴¹ reported that caspase-3 also recognized and interacted with actomyosin, titin, nebulin, and tropomyosin in myofibrillar protein and this interaction can trigger further hydrolysis. As a result, caspase-3 is vital in enhancing the tenderness of bovine muscle during aging. In the present study, the bovine muscle tenderness was higher in the fast pH decline group than in the slow group at 12–168 h postmortem and matched the caspase-3 activity. Hwang and Thompson⁷ found that bovine *longissimus dorsi* with lower pH before the onset of rigour mortis produced more tender meat in the later stages of aging, which backed up the current findings. Given the essential role of pH decline rate in caspase-3 activation, it is possible that fast pH decline increases bovine muscle tenderization via the mitochondrial apoptotic pathway. Our experiment also observed that the tenderness of the fast group was lower than that of the slow group at 6 h postmortem, which could be associated to calpain-1 activity. Previous research has revealed that calpain-1 activation occurred around 6 h postmortem in bovine muscle⁴⁸, and that calpain-1 degradation activity on the myofibrillar protein was strongest at pH 7.5⁴⁹. Lomiwesa et al.⁵⁰ concluded that although calpain-1 autolysis was fast at a low pH, the hydrolysis of myofibrillar proteins was diminished in pork muscle. Despite the fact that no experiments involving calpain-1 activity were conducted in this study, it can be hypothesized, based on previous studies, that the fast pH decline group may have decreased calpain-1 activity at 6 h due to the lower pH microenvironments. Therefore, various processes other than mitochondrial apoptosis may also be involved in the regulation of postmortem pH decline rate on bovine muscle tenderness during aging, and this needs to be investigated further in the future. The current study is simply a first step towards understanding the role of postmortem pH in muscle tenderization during aging and we believe our findings will encourage more research in this field.

Table 2

Changes in the myofibril fragmentation index (MFI) and shear force in bovine muscles of different glycolytic rates at 0.5, 6, 12, 24, 72, 120, and 168 h postmortem.

Indicators	Glycolytic rate	Postmortem aging time						
		0.5 h	6 h	12 h	24 h	72 h	120 h	168 h
MFI	Fast	64.07±7.11 ^F	65.13±6.53 ^{F*}	108.67±5.52 ^{E*}	125.60±7.63 ^{D*}	142.53±6.11 ^{C*}	167.40±6.77 ^{B*}	183.47±7.04 ^{A*}
	Slow	66.07±6.03 ^g	79.06±6.80 ^f	93.53±6.11 ^e	108.73±7.71 ^d	128.72±6.74 ^c	149.33±7.63 ^b	166.87±6.93 ^a
Shear force (kgf)	Fast	6.62±0.37 ^D	7.79±0.53 ^{B*}	8.45±0.33 ^{A*}	7.18±0.42 ^{C*}	6.07±0.40 ^{E*}	5.61±0.52 ^{EF*}	5.12±0.31 ^{F*}
	Slow	6.53±0.63 ^d	7.20±0.39 ^c	8.96±0.35 ^a	7.74±0.37 ^b	6.91±0.41 ^{cd}	6.24±0.34 ^{de}	5.72±0.35 ^e

A-B: Each letter denotes a statistically significant difference in the fast group at different postmortem times ($P<0.05$). a-c: Each letter denotes a statistically significant difference in the slow group at different postmortem times ($P<0.05$). * indicates the significance compared the same time points between two groups ($P<0.05$). All measurements were expressed as the mean ± SE.

Materials And Methods

Muscle sampling and preparation. Fifty crossbred bulls (Luxi yellow cattle × Simmental) aged 24 to 36 months with a bodyweight of 450±50 kg were randomly chosen from Gansu Kangmei Modern Agriculture and Animal Husbandry Industry Group Co., Ltd. (Linxia City, Gansu province, China). The protocol and procedures used were reviewed and approved by the Operating Procedures of Cattle Slaughter in the National Standards of China. The *Longissimus thoracis* in the left side of carcasses were removed after slaughter and stored at 4°C. The pH of the muscles were measured at 0.5, 2, 6, 12, 24, 48, and 72 h during aging. Based on the muscle pH at 0.5 h (Noted as pH0.5h) and 6 h (Noted as pH6h) postmortem, muscle samples from 12 carcasses were selected and divided into 2 groups (6 per group): a fast decreasing pH group (Noted as a fast group with pH6h < 5.8) and a slow decreasing pH group (Noted as a slow group with pH6h > 6.2)²⁰. The time point for each sample storage was 0.5, 6, 12, 24, 72, 120, and 168 h at 4°C, at which samples were collected and the muscle's shear force was measured immediately. A portion of samples was stored in liquid nitrogen for biochemical indicators analysis.

Determination of the muscle pH. pH was measured using a portable pH meter (Testo® 230 meter, Testo GmbH & Co., Lenzkirch, Germany). The electrode was calibrated with standard buffer solutions at 4.0 and 7.0 pH values (Mallinckrodt Chemicals, Phillipsburg, NJ, USA). The electrode was placed into the muscle at random locations throughout the muscle for measurement. Before the next measurement, the electrode probe was cleaned with ultrapure water (pH 7.0) and wiped dry with filter paper. Each sample was measured three times.

Differential Scanning Calorimetry (DSC) analysis. A PerkinElmer differential scanning calorimeter (Waltham, USA) was used to analyze the denaturation temperature of bovine muscle. Minced muscle tissue (10–15 mg) was enclosed in an aluminum box and heated from 20°C to 100°C at a rate of 3°C/min, using the sealed empty box as a reference. Denaturation temperature (T , °C) was calculated by Pyris-12 software (Perkin Elmer-Instruments, USA).

Determination of reactive oxygen species (ROS). Muscle ROS levels were determined according to a previously described method²¹ with some modifications. Briefly, muscle samples were fixed in 4% paraformaldehyde for 48 h and were trimmed, dehydrated in ethanol, followed by embedded in paraffin before being section into 4.0 µm thickness tissue slices. Tissue slices were dewaxed in xylene and anhydrous ethanol for 10 min, respectively. After being washed with PBS buffer (pH 7.2), the sections were incubated in 5 µmol/L DCFH-DA at 37°C for 60 min protected from light and were subsequently washed off with PBS. Finally, the slices were sealed with an anti-fluorescence quenching sealer and placed under a fluorescence microscope (CKX31, Olympus, Japan) for observation (Ex=490 nm and Em=520 nm). Sections were scanned (400×) by a panoramic scanner (CaseViewer 2.4, Hungary) and the average fluorescence intensity of each cell cross-section was analyzed using the Halo v3.0.311.314 software (Indicalabs, USA).

Extraction of muscle mitochondria. The extraction of mitochondrial and cytoplasmic proteins from muscle tissue was followed as a previously described method²². In brief, approximately 5.0 g of muscle samples were mixed with 50 mL of mitochondrial separation medium (1 mmol/L EDTA, 300 mmol/L sucrose, 0.5% bovine serum albumin, 15 mmol/L Tris, pH 7.2) and homogenized in an ice bath. The homogenate was centrifuged at 4°C at 1,000 × g for 10 min. The obtained supernatant was centrifuged at 4°C at 8,000 × g for 20 min. The supernatant contained the cytoplasmic protein in solution while the precipitate was then extracted in mitochondria. The resulting precipitate was mixed with buffer (containing 300 mmol/L sucrose, 1 mmol/L EDTA, 15 mmol/L Tris, pH 7.2) to serve as the mitochondrial solution. The biuret method was employed to measure the final protein concentration and the bovine serum albumin (Sigma Chemical Co., St. Louis, MO) was used as a standard.

Determination of mitochondrial membrane permeability. Mitochondrial membrane permeability was measured according to a previously described method¹³. The concentration of the mitochondrial solution was adjusted to 2 mg/mL and incubated with four times of volumes of cold test medium (including 250 mmol/L sucrose, 10 mmol/L Tris-HCl, pH 7.4) at room temperature for 3 min. Mitochondrial membrane permeability was determined

by analyzing the light absorption value of the mixed solution with a UV spectrophotometer at 520 nm. The lower absorbance indicated the higher mitochondrial permeability.

Determination of cytochrome c redox state. The method to determine cytochrome c redox was based on a previously described method²⁴ with minor modifications. Approximately 4.0 g of chopped muscle tissue was homogenized in 16.0 mL of cold separation buffer (0.3 mol/L sucrose, 0.5% bovine serum albumin, 1 mmol/L EDTA, 0.01 mol/L Tris-HCl, pH 7.4) and centrifuged at 4 °C at 1,000 × *g* for 10 min. After that, the supernatant was centrifuged at 4 °C at 8,000 × *g* for 15 min. The cytochrome c reduction levels were measured as the absorbance value of the obtained supernatant at 550 nm minus the absorbance value at 535 nm then divided by total protein concentration ($\Delta A_{550-535}/\text{mg protein}$). The biuret method was employed to measure the final protein concentration and the bovine serum albumin (Sigma Chemical Co., St. Louis, MO) was used as a standard.

Determination of caspase activity. A caspase-3 activity assay kit (Sigma-Aldrich, USA) was employed to determine its activity. In short, samples (1.0 g) were lysed in a precooled lysis solution (1 mmol/L dithiothreitol, 100 mmol/L Hepes, 0.1% NP-40 and 10% sucrose, pH 7.5). Samples were then smashed by employing a glass pestle and mortar 30 times under ice, and then the mixtures were centrifuged at 12,000 × *g* for 10 min. Subsequently, 45 µL of 10 mmol/L dithiothreitol and 5 µL of 4 mmol/L DEVD-pNA substrate were added into 20 µL of sample aliquots. Then the mixed solution was incubated at 37°C for 1 h and a fluorescence spectrophotometer (Shimadzu RF 5301, Kyoto, Japan) was used to measure the fluorescent intensity of the sample (Ex=360 nm and Em=460 nm).

Determination of apoptotic nucleus. TdT-mediated dUTP nick-end labeling (TUNEL) assay was used to detect the apoptotic nucleus. Muscle samples were cut into a block of 1.0 cm×0.5 cm×0.5 cm and fixed in a 4% paraformaldehyde solution for more than 48 h. Samples were paraffin-embedded and cut into ten µm-thickness tissue sections and transferred onto slides. After dewaxing and rehydration, the sections were immersed with formaldehyde solution containing 3% H₂O₂ to remove endogenous peroxidase. The sections were permeabilized by immersing them in a solution containing 0.1% Na₂NO₃ and 0.1% Triton X-100. The permeabilized tissue sections were rinsed and washed with buffer (137 mmol/L NaCl, 4.3 mmol/L disodium phosphate, 2.7 mmol/L KCl, 1.4 mmol/L potassium dihydrogen phosphate, pH 7.4). After washing, the sections were blocked by 5% goat serum for 30 min and then washed with the above buffer for 30 min. The labeling enzyme and the solution were prepared in a ratio of 1:9 to achieve the TUNEL reaction solution. The tissue sections were then covered with the solution prepared and incubated at 37°C for 60 min in a light protecting chamber. As for the negative controls, we used the lack of labeling enzyme during the TUNEL solution preparation. Positive controls were incubated with 5.0 U/mL DNase I for 10 min at room temperature before adding the TUNEL reaction solution. DAPI was added for nuclei staining, and the tissue sections were applied with glycerol and placed under a fluorescent microscope (CKX31, Olympus, Japan) for observation. Regular cell nuclei were stained in blue fluorescence by DAPI, and apoptotic cell nuclei were stained in green fluorescence by TUNEL. At least three fields of view per section were chosen and quantified for each sample (400×), and the apoptotic rate was calculated as the ratio of apoptotic nuclei to regular nuclei.

Determination of the myofibril fragmentation index (MFI). MFI was determined by the method as reported previously². In short, 5.0 g of meat was added with 20 mL of MFI buffer (100 mmol/L KCl, 20 mmol/L K₃PO₄, 1 mmol/L EDTA, 1 mmol/L MgCl₂, 1 mmol/L Na₃, pH 7.1) and homogenized on ice. The homogenate was then centrifuged at 4°C at 1000×*g* for 15 min. After that, the supernatant was discarded. Next, 20 mL of MFI buffer was used to re-suspend the precipitate and the samples were homogenized again. The mixture was then centrifuged to discard the supernatant. Afterward, the precipitate was dissolved with 10 mL of MFI buffer, and was filtered through a 200-mesh nylon screen. Another 5 mL of MFI buffer was added to help the myofibrillar protein pass through the filter. Filter myofibrillar protein was quantified via the biuret method to determine its protein concentration, and its final concentration was achieved at 0.5 mg/mL via additional MFI buffer. A UV spectrophotometer was used to measure the absorbance at 540 nm. The final value was obtained by multiplying the reading 200 times to represent the MFI.

Determination of the shear force. The shear force of bovine muscle was measured according to a previously described method¹⁴. The samples were cooked in a bag and submerged in water at 80°C until the central temperature reached above 70°C for 30 min. Subsequently, the meat samples were cooled to room temperature, and three to five meat columns (Φ 1.27) were removed along the direction of the muscle fibers as representative samplers. Each sample was then cut by a blade perpendicular to the long axis of the muscle fibers and the shear force was measured by using a tenderness meter (C-LM4, Harbin, China).

Statistical analysis. The data were analyzed using IBM SPSS software 21.0 (SPSS, Inc., Chicago, IL, USA). The results were presented as mean ± standard error (SE) from six biological replicates. The one-way analysis of variance (ANOVA) procedure followed by the Duncan's multiple range tests was used to separate the means. If *P*<0.05, the difference was significant. The student's t-test was employed to compare the differences in the means between the two groups (**P*<0.05, ***P*<0.01). The results were presented as mean ± standard error (SE).

Conclusion

The present study investigated the effect of postmortem pH decline rates on mitochondrial apoptosis and tenderness in bovine muscle. The results demonstrate that the fast pH decline promoted sarcoplasmic protein denaturation and ROS accumulation, which further enhanced mitochondrial dysfunction and cytochrome c oxidation, thereby improving postmortem bovine skeletal muscle tenderness via caspase-3 activation. Therefore, the fast pH decline can increase bovine muscle tenderization by inducing mitochondrial apoptosis, and postmortem pH decline rates are important in

ultimate muscle tenderness development throughout aging. This new observation will provide scientific insight about the involvement of postmortem pH changes in apoptosis and beef tenderization.

Declarations

Data availability

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

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Author contributions

J.M., L.H., and Q.Y. conceived and designed the experiments. J.M. and M.H. performed the experiments. J.M., L.H. analyzed the data. Q.Y. contributed reagents, materials, and analysis tools. J.M. and L.H. wrote and revised the paper. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

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Figures

Figure 1

The pH in bovine muscles of different glycolytic rates at 0.5, 2, 6, 12, 24, 48, and 72 h postmortem. A-D: Each letter denotes a statistically significant difference in the fast group at different postmortem times ($P < 0.05$). a-e: Each letter denotes a statistically significant difference in the slow group at different postmortem times ($P < 0.05$). ** and * indicate the significance compared the same time points between two groups (** $P < 0.01$, * $P < 0.05$). Bars represent the standard error of mean.

Figure 2

(A) Representative ROS immunofluorescence images of bovine muscles in the fast group (A1–C1) and the slow group (a1–c1) at 6, 12, and 24 h postmortem. (B) The average fluorescence intensity of ROS of muscle cell cross-sections in bovine muscles of different glycolytic rates at 6, 12, and 24 h postmortem. A–C: Each letter denotes a statistically significant difference in the fast group at different postmortem times ($P < 0.05$). a–c: Each letter denotes a statistically significant difference in the slow group at different postmortem times ($P < 0.05$). ** and * indicate the significance compared the same time points between two groups (** $P < 0.01$, * $P < 0.05$). Bars represent the standard error of mean.

Figure 3

The mitochondrial membrane permeability (A) and the Cytochrome c reduction levels (B) in bovine muscles of different glycolytic rates at 0.5, 6, 12, 24, 72, 120, and 168 h postmortem. A-E: Each letter denotes a statistically significant difference in the fast group at different postmortem times ($P < 0.05$). a-e: Each letter denotes a statistically significant difference in the slow group at different postmortem times ($P < 0.05$). * indicates the significance compared the same time points between two groups ($P < 0.05$). Bars represent the standard error of mean.

Figure 4

The caspase-3 activity in bovine muscles of different glycolytic rates at 0.5, 6, 12, 24, 72, 120, and 168 h postmortem. A-D: Each letter denotes a statistically significant difference in the fast group at different postmortem times ($P < 0.05$). a-e: Each letter denotes a statistically significant difference in the slow group at different postmortem times ($P < 0.05$). * indicates the significance compared the same time points between two groups ($P < 0.05$). Bars represent the standard error of mean.

Figure 5

(A) Representative TUNEL positive nucleus images of bovine muscles in the fast group (A1–F1) and the slow group (a1–f1) at 24, 72, 120, and 168 h postmortem. Normal nuclei were labeled with blue fluorescence by adopting 4,6-diamidino-2-phenylindole (DAPI) dyes, whereas the apoptotic nuclei were labeled with green fluorescence through TUNEL. (B) The apoptosis rate in bovine muscles of different glycolytic rates at 24, 72, 120, and 168 h postmortem. A–D: Each letter denotes a statistically significant difference in the fast group at different postmortem times ($P<0.05$). a–d: Each letter denotes a statistically significant difference in the slow group at different postmortem times ($P<0.05$). * indicates the significance compared the same time points between two groups ($P<0.05$). Bars represent the standard error of mean.