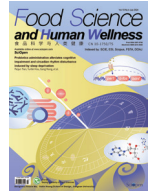




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Endoplasmic reticulum stress improved chicken tenderness, promoted apoptosis and autophagy during postmortem ageing

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ABSTRACT

In this study, endoplasmic reticulum (ER) stress inducer tunicamycin (TM) and inhibitor 4-phenylbutyric acid (4-PBA) were used to treat postmortem chicken breast muscle to investigate changes in tenderness and effects on apoptosis and autophagy during 5 days ageing. TM-induced ER stress reduced shear force, enhanced myofibril fragmentation index (MFI), disrupted myofibril structure, increased desmin degradation, and activated μ -calpain and caspase-12. In addition, TM-induced ER stress increased the expression of Bax, Bim, and cytochrome c, and decreased the expression of Bcl-xL. Furthermore, TM-induced ER stress improved the conversion of LC3I to LC3II, raised the expression of Beclin-1, and decreased the expression of p62, PI3K, and mTOR. The opposite results were observed after 4-PBA treatment. These results suggested that ER stress could improve chicken tenderness, promote apoptosis and autophagy during chicken postmortem ageing.

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1. Introduction

The quality of meat is an important indicator that influences consumers to purchase, among which tenderness is one of the most important indicators to determine the edible quality of meat^[1]. Tenderness is highly susceptible to change during the storage of meat compared to other quality evaluation indicators such as flavor and juiciness. The muscle within the animal tissue undergoes a series of changes in physicochemical properties after slaughter, mainly including rigor mortis and ageing^[2]. Muscles are generally stored at 0–4 °C for postmortem ageing. Postmortem ageing helps to transform inedible muscles into edible meat and is an effective way to improve meat tenderness^[3]. The main reason for the increase in meat

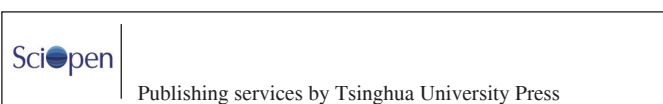
tenderness after slaughter is the degradation of myofibrillar proteins by endogenous proteases. Apoptotic enzymes and calpain are the most important enzymes that contribute to the transformation of muscle into edible meat, which degrade structural proteins ultimately causing the loss of the cytoskeleton^[4–5].

The contribution of apoptosis theory in meat ageing has received great attention in recent years^[6–7]. After slaughter, animals are drained of almost all blood in a very short time, and in such cases, the myocytes of the animal body are inevitably in a state of hypoxia and ischemia, leading to apoptosis and autophagy^[8]. Apoptosis refers to the cell receiving apoptotic signals, coordinating the activities of a series of apoptotic enzymes and apoptotic factors to transduce the signals, and finally completing the death of cells^[9]. Different apoptosis-inducing signals form the different transduction pathways, and the main transduction pathways found so far include death receptor pathway, mitochondrial pathway, and endoplasmic reticulum (ER) stress pathway^[10]. Mitochondria, as an important organelle of energy metabolism, mitochondrial apoptosis has shown to be beneficial to meat tenderization, in which the Bcl-2 family

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plays an important role^[11-12]. The Bcl-2 family control apoptosis and survival through the interaction of pro- and anti-apoptotic molecules. The ER produces most of the proteins and some of the sterols and lipids required by the organism and is an important organelle. When cells are subjected to sustained chemical stimulation, oxidative stress, or imbalance in energy metabolism, they undergo a series of changes. Abnormal protein synthesis in the ER, imbalance in calcium homeostasis, and dysfunction of the ER all lead to ER stress^[13]. Postmortem hypoxia and ischemia will damage the homeostasis of the internal environment, thus it can cause ER stress^[8]. However, whether ER stress plays a role in meat tenderization during postmortem ageing is unclear. Autophagy is also called type II programmed cell death. Autophagy is an important intracellular metabolic pathway in which misfolded proteins and damaged organelles are separated from the cytoplasm to form autophagosomes, and are transferred to lysosomes for degradation^[14]. Spanish scientists have identified autophagy in early postmortem beef^[15]. However, there are few studies related to ER stress and autophagy during postmortem meat tenderization. In addition, the influences of ER stress-mediated apoptosis and autophagy on meat quality have rarely been investigated.

Therefore, the goal of this study was to find whether ER stress affects tenderness, and further explore the effects of ER stress on apoptosis and autophagy during postmortem ageing by treating chicken muscle with the inducer of ER stress, tunicamycin (TM), and the inhibitor 4-phenylbutyric acid (4-PBA).

2. Materials and methods

2.1 Chemicals

TM and 4-PBA were bought from Sigma Aldrich (St. Louis, Mo, USA). Bicinchoninic acid (BCA) protein analysis kit was purchased from Thermo Scientific (MA, USA). RT mix with DNase (all in one) kit, 2 × SYBR Green qPCR Master Mix kit were purchased from US EVERBRIGHT Inc. (Suzhou, China). The TRIzol kit was purchased from Tianmo biotech (Beijing, China). Whole protease inhibitor cocktail tablet was purchased from Roche (Mannheim, Germany). Enhanced chemiluminescence (ECL) polyvinylidene fluoride (PVDF) membrane and western blotting detection reagents were obtained from Millipore (MA, USA). Antibodies against Bax (#2772), Bim (#2933), Bcl-xL (#2752), Beclin-1 (#3738), BiP (#3277), SQSTM1/p62 (#39749), CHOP (#2895), Calpain 1 Large Subunit (#2556), GAPDH rabbit MAb (#2118), HRP-linked anti-rabbit IgG, HRP-linked anti-mouse IgG were acquired from Cell Signaling Technology (MA, USA). Antibodies against IRE1 (ab37073), XBP1 (ab198999) and caspase-12 (ab62484) were procured from Abcam (Cambridge, UK).

2.2 Animals

Ten chickens (Shaanxi Sanhuang chickens, male, 45 days, 2.5–3.0 kg) were picked at random and bled for slaughter after being stunned for 5 s at 400 Hz using an electrical stunner (SQ805A, Jiangsu Wujiang Aneng Electronic Technology Co., Ltd., Suzhou, China) under 40 V AC conditions. This process was following the guidelines approved by the Institutional Animal Care and Use Committee of Northwest A&F University (approved ID: 2015-1-138).

2.3 Sample collection and treatment

Chicken breast muscles were quickly collected after slaughter, excised of fat and connective tissue, then divided into two parts for following experiments.

Chicken breast muscles were chopped, then immediately weighed about 6 g of meat samples, quickly frozen in liquid nitrogen, and stored at −80 °C as samples for 0 day. The remainder of chopped muscles were subdivided into three fractions and allocated to treatments as follows: one portion was soaked in 0.9% NaCl solution (control group) and the two other sections were soaked in solutions containing either 0.5 mg/mL TM, or 2.5 mg/mL 4-PBA, and then stored at 4 °C for 1, 3, and 5 days. The meat to solution ratio was 1:2 (*m/V*). Samples were taken at the corresponding time points and quickly frozen in liquid nitrogen, then stored at −80 °C for subsequent analysis.

2.4 Shear force measurements

The shear force was determined referring to the method of Wang et al.^[8] with some modifications. Chicken breast muscles were cut into 50 mm × 40 mm × 20 mm pieces along the fiber direction, randomly separated into three groups, and injected with 0.9% NaCl, 0.5 mg/mL TM, and 2.5 mg/mL 4-PBA solutions respectively according to the meat/solution ratio of 10:1 (*m/V*), then stored at 4 °C for 1, 3, and 5 days. The samples were collected at the corresponding time points and heated in vacuum bag at 75 °C water bath until the central temperature to 70 °C. After removal, the samples were cooled naturally to room temperature and divided into 40 mm × 10 mm × 10 mm strips parallel to the direction of the muscle fibers. The meat strips were measured with texture testing machine (Texture Analyzer TA.XT.plus, Stable Micro Systems, Surrey, UK) in the direction of the perpendicular muscle fibers, and the results were expressed as shear force (N).

2.5 Myofibril fragmentation index (MFI) determination

The determination of MFI has been modified from the method of Chen et al.^[16]. Added 16 mL MFI buffer (100 mmol/L KCl, 20 mmol/L K₃PO₄, 1 mmol/L MgCl₂, 1 mmol/L EDTA, 1 mmol/L NaN₃, pH = 7.1) to per gram meat sample, and homogenized at 15 000 r/min for 3 times for 10 s each time. The supernatant was discarded after centrifugation at 4 °C, 1 000 × g for 15 min, then added sixteen volumes buffer to the precipitation, mixed evenly, and repeated centrifugation once. The precipitates were resuspended in sixteen volumes buffer, then filtered the tissue fragments through a 200 mesh sieve. The protein concentration of the filtrate was measured with BCA protein analysis kit and diluted to 0.5 mg/mL with buffer. The absorbance was determined at 540 nm with ultraviolet spectrophotometer (UV-1780, Suzhou Shimadzu Instrument Co., Ltd., Suzhou, China). The absorbance multiplied by 200 was the MFI value.

2.6 Proteins extraction and preparation

For extraction of sarcoplasmic proteins, referred to the previous method^[17]. Briefly, 3 mL tissue lysis buffer (25 mmol/L Tris, 150 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L DTT, 5 mmol/L NaF, 1 mmol/L

PMSF, 1% Triton X-100 and protease mix inhibitor cocktail, pH = 7.6) was added to per gram meat sample, and the mixture was homogenized three times at 15 000 r/min for 10 s each. The samples were then centrifuged at 4 °C for 30 min at 15 000 × g. The supernatant was obtained and diluted uniformly after the determination of protein concentration using the BCA protein assay kit.

The purification procedure of myofibrillar proteins was referred to the previous method^[18]. Per gram meat sample was added with 7.5 mL PRB buffer (100 mmol/L KCl, 2 mmol/L MgCl₂·H₂O, 2 mmol/L EDTA-2Na, 1 mmol/L NaN₃, 2 mmol/L Na₄P₂O₇·10H₂O, 10 mmol/L maleate, pH = 6.8), and homogenized three times at 15 000 r/min for 10 s each. The supernatant was discarded and 7.5 mL PRB buffer without pyrophosphate was added to the per gram precipitate, homogenized at 15 000 r/min for 5 s, and then centrifuged at 1 000 × g for 10 min at 4 °C, repeated 3 times. The precipitate was added to 7.5 mL 15 mmol/L Tris (pH = 8.0), homogenized at 15 000 r/min for 5 s, and then centrifuged at 1 000 × g for 10 min at 4 °C, and repeated 2 times. The last precipitate was dissolved in Tris and diluted uniformly after the determination of protein concentration using the BCA protein assay kit.

Sample treatment solution (10% MCE, 20% glycerol, 0.001% bromophenol blue) was added to the sarcoplasmic and myofibrillar proteins and heated at 99 °C for 5 min. The prepared protein samples were used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting.

2.7 SDS-PAGE and western blotting analysis

SDS-PAGE was performed using 12.5% of the lower separating gel and 4% of the upper stacking gel. The electrophoresis instrument (Bio-Rad Laboratories, CA, USA) was set at a constant voltage and run at 80 V for 45 min, followed by 120 V for 70 min. After electrophoresis, the proteins on the gels were transferred onto PVDF membranes (Millipore, CA, USA) using Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, CA, USA) at a transfer voltage of 10 V. The transfer time was 18–27 min basing on the molecular weight of the target protein. Prepared the blocking solution of 5% skimmed milk powder with TBST buffer (200 mmol/L Tris, 300 mmol/L NaCl, 0.05% Tween-20, pH = 7.5), and placed the transferred PVDF membranes in the blocking solution with gentle shaking for 3 h. PVDF membranes were moved into the corresponding protein primary antibody (1:1 000) overnight at 4 °C. The membranes were washed with TBST to remove the residual primary antibody, placed in HRP-linked anti-rabbit IgG or HRP-linked anti-mouse secondary antibody (1:5 000), and incubated at room temperature for 2 h. The membranes were collected images with ECL by the Chemiluminescence imaging system after being washed with TBST. The Lane 1D software was used for the quantitative analysis of bands.

2.8 Transmission electron microscopy (TEM) analysis

Chicken breast muscles were cut into 1 mm³ parallel to the muscle fiber direction and placed in 2.5% glutaraldehyde fixative for immersion fixation. Then 0.1 mol/L phosphate buffer was washed 3–4 times for 15 min each time. 1% osmium acid was fixed and then washed with 0.1 mol/L phosphate buffer. Then after gradient dehydration in ethanol (30%, 50%, 70%, 80%, 90%, 100%), resin

infiltration was performed with different concentrations embedding agent, 25% for 2 h, 50% for 8 h, 75% for 12 h, and 100% at room temperature for 48 h. LR White was added to the capsules for embedding and polymerized at 55 °C for 48 h, followed by ultra-thin sectioning using an ultramicrotome (EMUC7, Leica, Austria) at a thickness of approximately 50–70 nm. The sections were stained with 2% uranium dioxide acetate for 10–30 min and lead citrate for 5–15 min. After rinsing and drying, sections were magnified by 13 000 times and were observed by TEM (TECNAI G2 SPIRIT BIO, FEI Co., USA).

2.9 Total RNA extraction and real-time quantitative PCR (RT-qPCR) analysis

The total RNA was extracted using the TRIzol kit and adjusted to uniform concentration after RNA quantification by NanoDrop2000 Micro Nucleic Acid Analyzer (Bio-Rad, CA, USA). cDNA was synthesized by reverse transcription with RT mix with DNase (all in one) kit according to the manufacturer's agreement. Using 2 × SYBR Green qPCR Master Mix Kit to combine fluorescent dyes with dsDNA then quantified by fluorescence quantitative PCR (Quant Studio 6, Life Technologies, Carlsbad, USA). PCR cycling conditions were 95 °C for 120 s, followed by 45 cycles at 95 °C for 5 s and 60 °C for 30 s. The gene expression was analyzed by the 2^{−ΔΔCt} method. The primer sequences used are shown in Table 1.

Table 1
Primer sequences used for RT-qPCR analysis.

Gene	Primer sequences(5' to 3')
<i>GAPDH</i>	Forward: CAGCCAGCCATGGATGATGA Reverse: ACCAACCATCACACCTGTAT
<i>cyto-c</i>	Forward: CTTACACAGATGCCAAT Reverse: AGACTTCTTCTTGATACC
<i>Bcl-xL</i>	Forward: CTTTCAGCGACCTCACCTC Reverse: ACAATGCGTCCCACCAGT
<i>LC3I</i>	Forward: GCTGGACAAGACCAAGTTCC Reverse: TTCACCAGCAGGAAGAAAGC
<i>LC3II</i>	Forward: ATCCGAGATCAGCATCCAAC Reverse: GTGATCTGGCACCAGGAAC
<i>mTOR</i>	Forward: GGACTCTTCCTGTGGCTAA Reverse: TACGGGTGCCCTGGTTCTG
<i>PI3K</i>	Forward: GTCCTTGAGCCACTGATG Reverse: TGTTCGCTTACGGTTGTT

2.10 Statistical analysis

All data were expressed as mean ± SE and analyzed by one-way ANOVA using the statistical program SPASS 20.0. Duncan's multiple range tests were used to compare differences among the means of each group. *P* < 0.05 considered differences significant, *P* < 0.01 considered extremely significant.

3. Results

3.1 Effect of TM and 4-PBA treatment on ER stress

First, chicken muscles were treated with the ER stress inducer TM and the inhibitor 4-PBA to verify their effects on ER stress. The IRE1 signaling pathway is an important ER stress pathway. The expression

of XBP1, IRE1, BiP, and CHOP bands were enhanced after TM treatment compared with the control group (Figs. 1A-E). On the contrary, the intensity of XBP1, IRE1, BiP, and CHOP bands reduced after 4-PBA treatment and were extremely significant on day 1 and day 3 (Figs. 1F-J, $P < 0.01$). The results showed that TM treatment activated the IRE1 signaling pathway and induced the expression of ER stress-related proteins, causing the activation of ER stress, while 4-PBA treatment attenuated the expression of ER stress-related proteins, resulting in the inhibition of ER stress. Thus TM and 4-PBA treatments could effectively model ER stress.

3.2 Effect of ER stress on shear force and MFI

The tenderness of the meat is usually assessed by shear force. Shear force tended to decrease gradually with increasing postmortem

ageing time (Figs. 2A and B). Shear force was extremely significantly lower after TM treatment than in the control group on day 1 and day 3 (Fig. 2A, $P < 0.01$). While 4-PBA treatment increased shear force significantly on day 1 (Fig. 2B, $P < 0.05$) and extremely significantly on day 3 (Fig. 2B, $P < 0.01$).

MFI is an indicator of the degradation degree of myofibrils and has a connection with tenderness^[1]. TM treatment significantly increased MFI values compared to the control group, with extremely significant differences between day 1 and day 3 (Fig. 2C, $P < 0.01$), while 4-PBA reduced MFI values with extremely significant differences between day 1 and day 5 (Fig. 2D, $P < 0.01$). Therefore, TM-induced ER stress damaged the internal structural integrity of myofibrils, while 4-PBA-inhibited ER stress reduced the damage. These results suggested that ER stress improved tenderness during postmortem ageing.

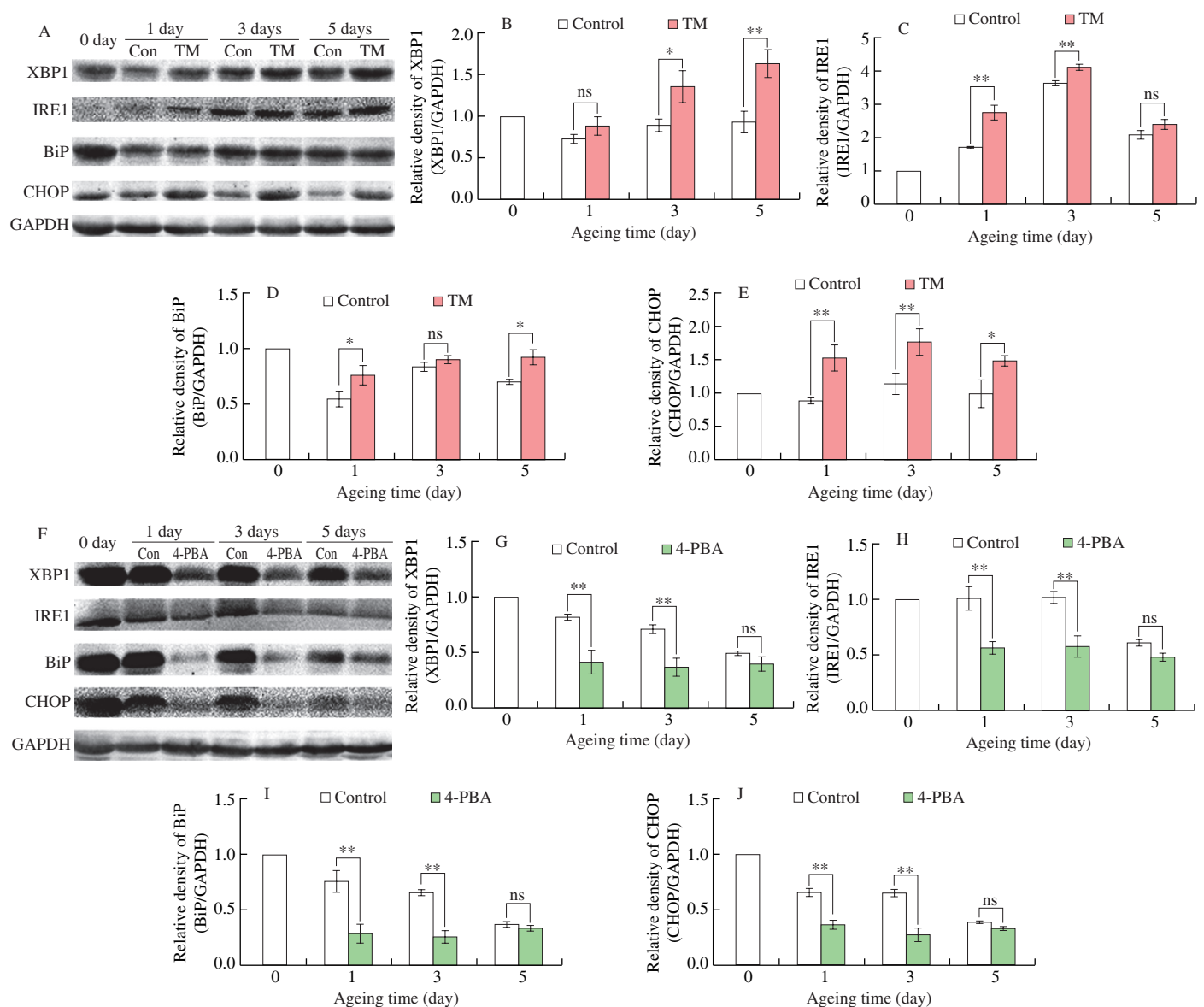


Fig. 1 Effect of TM and 4-PBA treatment on ER stress pathway during postmortem ageing of chicken muscle. (A) Western Blotting analysis of XBP1, IRE1, BiP, and CHOP on days 1, 3, and 5 after treated with 0.5 mg/mL TM. Densitometric analysis of XBP1 (B), IRE1 (C), BiP (D), and CHOP (E). (F) Western Blotting analysis of XBP1, IRE1, BiP, and CHOP on days 1, 3, and 5 after treated with 2.5 mg/mL 4-PBA. Densitometric analysis of XBP1 (G), IRE1 (H), BiP (I), and CHOP (J). Relative values were calculated as the ratio of corresponding ageing time density to day 0 and then to internal control. Mean $\pm$ SE ($n = 3$). * $P < 0.05$, ** $P < 0.01$.

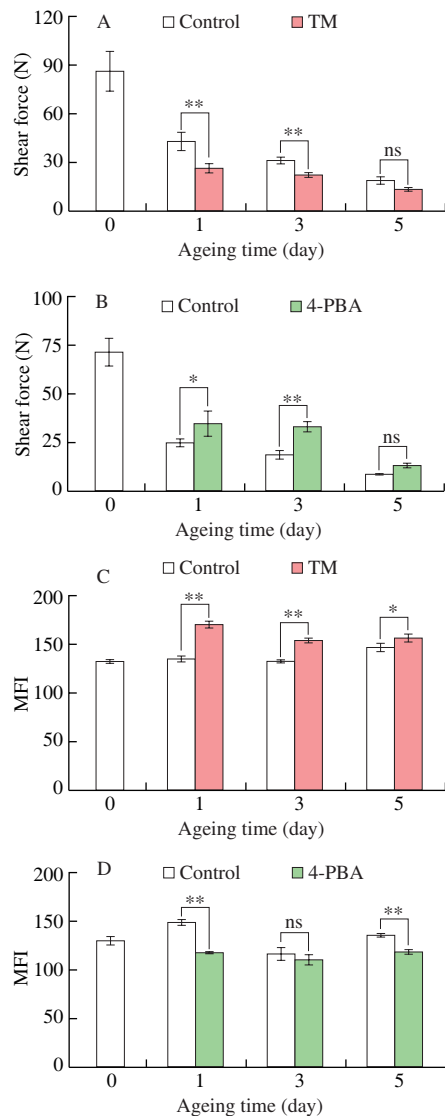


Fig. 2 Effect of ER stress on shear force and MFI during postmortem ageing of chicken muscle. Shear force of (A) 0.5 mg/mL TM and (B) 2.5 mg/mL 4-PBA treatment during postmortem ageing. MFI of (C) 0.5 mg/mL TM and (D) 2.5 mg/mL 4-PBA treatment during postmortem ageing. Mean $\pm$ SE ($n = 4$). * $P < 0.05$, ** $P < 0.01$.

3.3 Effect of ER stress on ultrastructure of myofibrils

The ultrastructure of myofibrils in the longitudinal section was observed by transmission electron microscopy. The structure of myofibrils was intact on day 0, and Z-lines were visible (Fig. 3O). In the control group, A-bands and I-bands were relatively easy to distinguish and the structure was changed, but Z-lines were relatively complete (Figs. 3A-C), whereas the structure of myofibrils was significantly changed and severely damaged after TM treatment (Figs. 3G-I). Broadening of I-bands was observed on day 1 of TM treatment (Fig. 3G), which occurred on day 3 of the control group (Fig. 3B), while it was not observed on day 5 of 4-PBA treatment (Fig. 3F). I-bands were slightly broken in the control group (Figs. 3A-C), whereas breakage of I-bands were more severe in TM treatment (Figs. 3G-I). In the control group, slight degradation of M-lines started on day 3 (Fig. 3B) and occurred on day 5 of 4-PBA treatment (Fig. 3F), while this phenomenon was observed on day 1 of TM treatment and

the boundaries between A-bands and I-bands started to blur (Fig. 3G). On day 5 of TM treatment, M-lines and A-bands almost completely disappeared, I-bands were severely disintegrated, the myofibrillar skeleton was almost completely degraded, junctions of adjacent myofibrils were also broken, and Z-lines were relatively blurred and distorted (Fig. 3I). Compared with the control group, except for the degradation of M-lines on day 5, the structure of myofibrils was relatively intact after 4-PBA treatment, boundaries of A-bands and I-bands were significant, and Z-lines were clear and intact (Figs. 3D-F). These results indicated that TM-induced ER stress had a great effect on myofibrils, leading to the disintegration of myofibrils by breaking down the main structure, while on the contrary, 4-PBA inhibited ER stress, attenuated the degradation of myofibrils, and played a protective role on myofibrils structure.

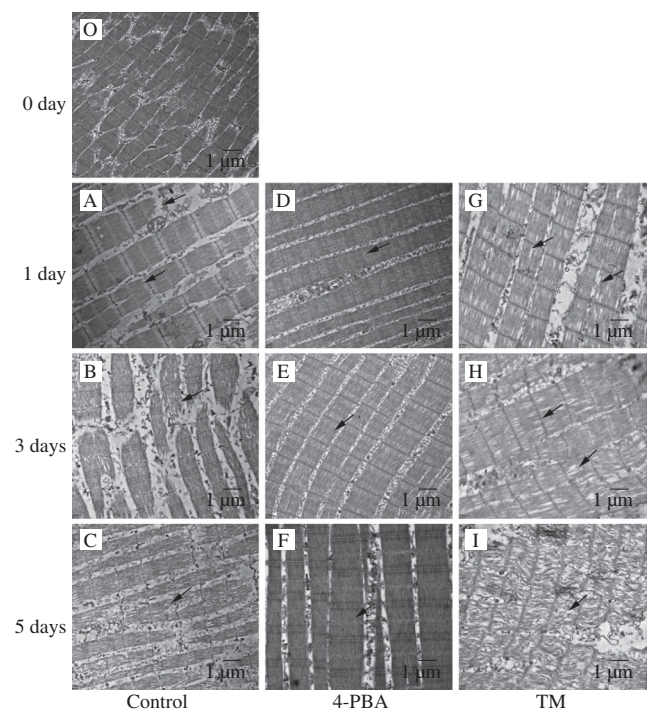


Fig. 3 Effect of ER stress on ultrastructure of myofibrils in longitudinal section during postmortem ageing of chicken muscle. (O, A, B, C) Control groups; (D, E, F) 2.5 mg/mL 4-PBA-treated groups; (G, H, I) 0.5 mg/mL TM-treated groups. Arrows showed breakage of myofibrils. 13 000 $\times$ magnification, scale bar = 1 μ m.

3.4 Effect of ER stress on desmin degradation and endogenous enzymes

The degradation of key myofibrillar proteins can increase meat tenderness. Desmin is an intermediate filament protein in the myofibrillar skeleton, and degradation of desmin has a positive effect on increasing tenderness. The intensity of desmin bands were extremely significant reduced in the TM-treated group on day 1 and day 5 compared to the control group (Figs. 4A and B, $P < 0.01$). While in contrast, 4-PBA treatment extremely significantly increased the intensity of desmin bands (Figs. 4A and C, $P < 0.01$). These indicated that the degradation of desmin was aggravated by TM-induced ER stress, while 4-PBA-inhibited ER stress protected the degradation of desmin.

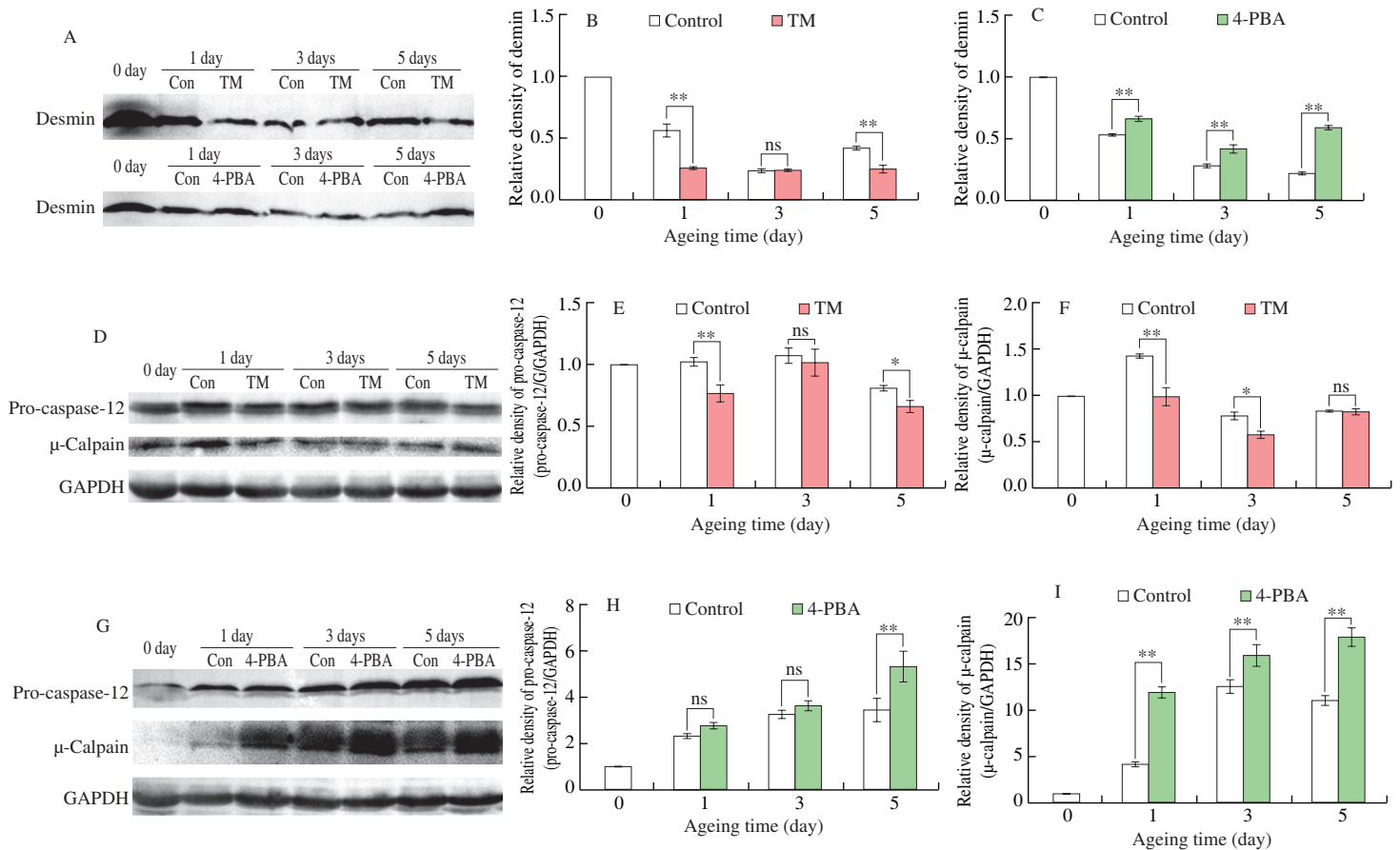


Fig. 4 Effect of ER stress on desmin degradation and endogenous enzymes during postmortem ageing of chicken muscle. (A) Western blotting analysis of desmin with 0.5 mg/mL TM and 2.5 mg/mL 4-PBA on days 1, 3, and 5. Densitometric analysis of desmin with (B) TM and (C) 4-PBA. (D) Western blotting analysis of pro-caspase-12 and μ -calpain during postmortem ageing on days 1, 3, and 5 after treated with 0.5 mg/mL TM. Densitometric analysis of (E) pro-caspase-12 and (F) μ -calpain. (G) Western blotting analysis of pro-caspase-12 and μ -calpain at 1, 3, and 5 days after treated with 2.5 mg/mL 4-PBA. Densitometric analysis of (H) pro-caspase-12 and (I) μ -calpain. Relative values were calculated as the ratio of corresponding ageing time density to day 0 and then to internal control. Mean $\pm$ SE ($n = 3$). * $P < 0.05$, ** $P < 0.01$.

The traditional theory of meat ageing has considered that μ -calpain is the most important endogenous enzyme during postmortem ageing. Caspase-12 is the only caspase family member located in the ER, which is in the form of inactive pro-caspase-12, and the activation is due to ER stress^[19]. The intensity of the 80 kDa large subunit bands of μ -calpain after TM-induced ER stress was significantly lower on day 1 and day 3 (Fig. 4D and F, $P < 0.05$), and pro-caspase-12 expression was significantly lower on day 1 and day 5 than that of the control group (Figs. 4D and E, $P < 0.05$), indicating activation of μ -calpain and caspase-12. The intensity of μ -calpain bands was extremely significantly increased after 4-PBA-inhibited ER stress (Fig. 4G and I, $P < 0.01$), and pro-caspase-12 was extremely significantly increased on day 5 (Figs. 4G and H, $P < 0.01$), indicating that μ -calpain and caspase-12 were inhibited.

3.5 Effect of ER stress on apoptosis

The Bcl-2 family plays a considerable role in the mitochondrial pathway of apoptosis. The expression of Bcl-2 family anti-apoptotic factor Bcl-xL protein and mRNA were decreased after TM-induced ER stress (Figs. 5A, D and F), while the pro-apoptotic factor Bax protein intensity was significantly enhanced (Figs. 5A and B, $P < 0.05$) and Bim protein intensity was extremely significantly increased

on day 3 and day 5 (Fig. 5C, $P < 0.01$). The expression of *cyto-c* mRNA increased extremely significantly on day 1 ($P < 0.01$) and significantly on day 3 of TM treatment (Fig. 5E, $P < 0.05$), indicating that mitochondrial membrane permeability was affected. In contrast, the expression of Bcl-xL protein and mRNA in the 4-PBA-treated group increased significantly on day 3 and day 5 (Figs. 5G, J and L, $P < 0.05$), and the intensity of Bax and Bim protein was lower than that of the control group, and the effect was most significant on day 5 (Figs. 5G-I, $P < 0.01$). The expression of *cyto-c* mRNA decreased extremely significantly on day 1 ($P < 0.01$) and significantly on day 5 (Fig. 5K, $P < 0.05$). It showed that TM-induced ER stress promoted apoptosis by activating the pro-apoptotic factors and inhibiting the anti-apoptotic factor, while 4-PBA-inhibited ER stress had the opposite effect.

3.6 Effect of ER stress on autophagy

Microtubule-associated protein light chain 3 (LC3) is a necessary marker for autophagy occurrence, and LC3II content is proportional to the number of autophagic vacuoles, which is a criterion for determining autophagy occurrence. The expression of *LC3III/LC3I* mRNA tended to increase after TM treatment and was extremely significant higher than that of the control group on day 3 (Fig. 6E,

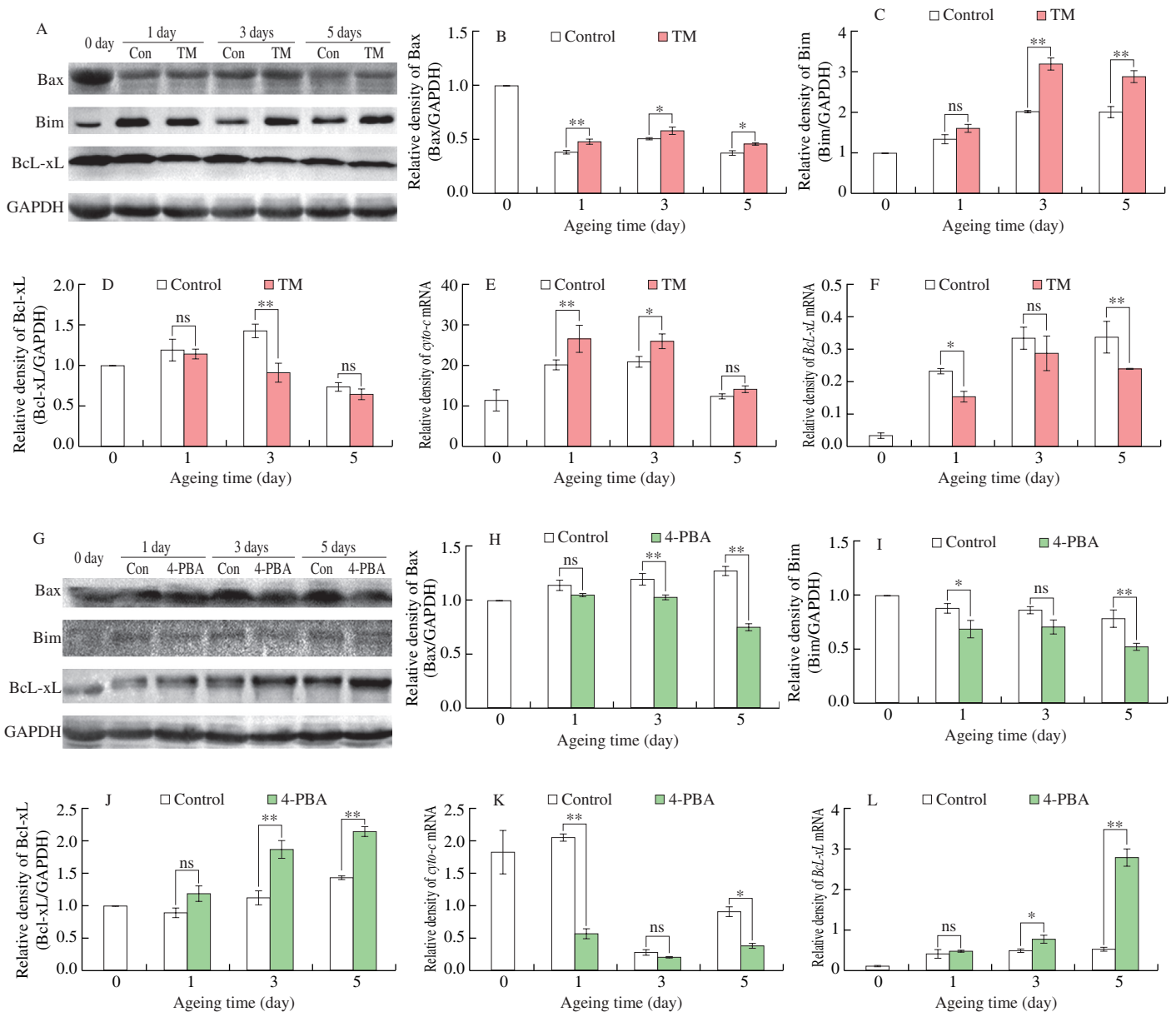


Fig. 5 Effect of ER stress on Bcl-2 family during postmortem ageing of chicken muscle. (A) Western blotting analysis of Bax, Bim, and Bcl-xL on days 1, 3, and 5 after treated with 0.5 mg/mL TM. Densitometric analysis of (B) Bax, (C) Bim, and (D) Bcl-xL. Quantitative mRNA analysis of (E) *cyto-c* and (F) *Bcl-xL*. (G) Western blotting analysis of Bax, Bim, and Bcl-xL on days 1, 3, and 5 after treated with 2.5 mg/mL 4-PBA. Densitometric analysis of (H) Bax, (I) Bim, and (J) Bcl-xL. Quantitative mRNA analysis of (K) *cyto-c* and (L) *Bcl-xL*. Relative values were calculated as the ratio of corresponding ageing time density to day 0 and then to internal control. Mean $\pm$ SE ($n = 3$). * $P < 0.05$, ** $P < 0.01$.

$P < 0.01$), indicating that LC3II was activated, whereas the expression of *LC3III/LC3I* mRNA was lower in the 4-PBA-treated group (Fig. 6J), and the transition from LC3I to LC3II was inhibited. Beclin-1 is an important protein in autophagy development and regulation. The increase in Beclin-1 protein expression was extremely significant after TM treatment compared to the control group (Figs. 6A, B, $P < 0.01$), while the intensity of beclin-1 bands reduced extremely significantly on day 3 and day 5 after 4-PBA treatment (Figs. 6G and H, $P < 0.01$). p62 is a negative regulator of autophagy. The p62 protein expression reduced after TM treatment, and the difference was extremely significant on day 1 (Figs. 6A and D, $P < 0.01$), while it was extremely significantly increased on day 3 and day 5 of 4-PBA treatment (Figs. 6G and I, $P < 0.01$). These results suggested

that autophagy was activated by TM-induced ER stress and inhibited by 4-PBA-inhibited ER stress.

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that is involved in a variety of biological processes in cells and is a key autophagic regulator. mTOR signaling pathway has a great influence on autophagy, and Class I phosphoinositide-3-kinase (PI3K) is upstream of mTOR. The expression of *PI3K* mRNA was lower than that of the control group after TM treatment, the differences were extremely significant (Fig. 6F, $P < 0.01$), and the protein expression of mTOR reduced significantly on day 3 and day 5 (Figs. 6A and C, $P < 0.05$). While 4-PBA extremely significantly increased the expression of *PI3K* mRNA on day 3 and day 5 (Fig. 6K, $P < 0.01$), and extremely significantly increased

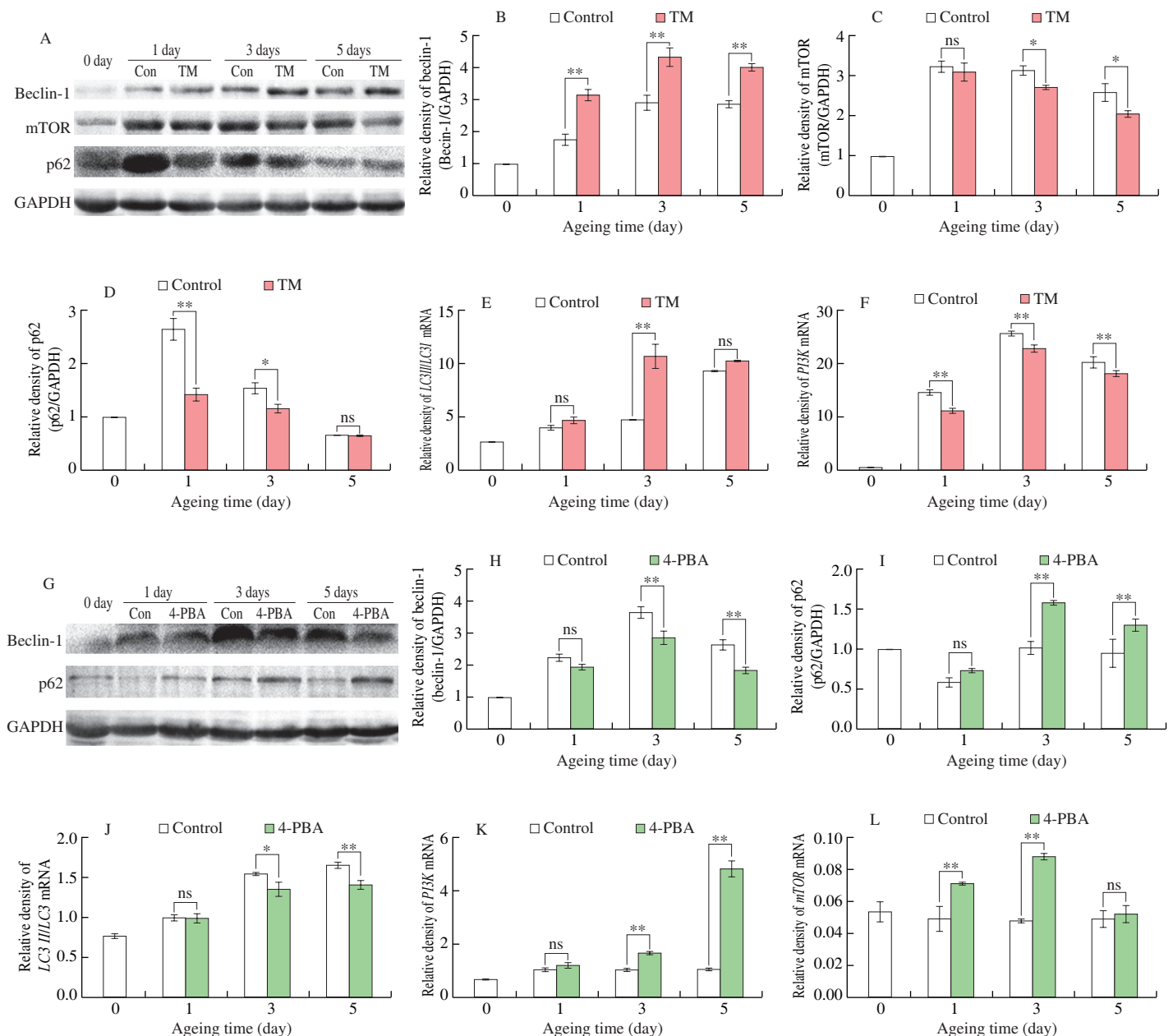


Fig. 6 Effect of ER stress on autophagy during postmortem ageing of chicken muscle. (A) Western blotting analysis of beclin-1, mTOR, and p62 during postmortem ageing on days 1, 3, and 5 after treated with 0.5 mg/mL TM. Densitometric analysis of (B) beclin-1, (C) mTOR, and (D) p62. Quantitative mRNA analysis of (E) *LC3III/LC3I* and (F) *PI3K*. (G) Western blotting analysis of beclin-1 and p62 during postmortem ageing on days 1, 3, and 5 after treated with 2.5 mg/mL 4-PBA. Densitometric analysis of (H) beclin-1 and (I) p62. Quantitative mRNA analysis of (J) *LC3III/LC3I*, (K) *PI3K*, and (L) *mTOR*. Relative values were calculated as the ratio of corresponding ageing time density to day 0 and then to internal control. Mean $\pm$ SE ($n = 3$). * $P < 0.05$, ** $P < 0.01$.

the expression of *mTOR* mRNA on day 1 and day 3 (Fig. 6L, $P < 0.01$). These have shown that ER stress regulated autophagy may be associated with mTOR during chicken postmortem ageing.

4. Discussion

This study aimed to investigate the role of ER stress on postmortem chicken muscle tenderization and the regulation of apoptosis and autophagy, through the inducer TM and the inhibitor 4-PBA of ER stress.

Under normal physiological conditions, glucose regulated protein 78 (GRP78/BiP), a signature protein of ER stress, binds to 3

receptors on the ER membrane, namely pancreatic ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1), rendering them inactivated. Upon the occurrence of ER stress, BiP dissociates from these 3 receptor proteins, activating 3 independent signaling pathways^[20]. IRE1 has protein kinase and RNA endonuclease activities, and when ER stress occurs, activated IRE1 splices *XBP1* mRNA, which further translates to produce XBP1, a transcription factor that upregulates target genes. As one of the C/EBP family of transcription factors, C/EBP-homologous protein (CHOP) is a signature gene for apoptosis in ER stress and is involved in ER stress-regulated apoptosis^[21]. Continuous and intense ER stress could activate CHOP in large amounts. In the present study, TM

treatment upregulated the expression of BiP, IRE1, XBP1, and CHOP (Figs. 1A–E), demonstrating that TM induced ER stress in postmortem ageing through activation of the IRE1 pathway and might be involved in regulating apoptosis. In contrast, 4-PBA treatment inhibited ER stress (Figs. 1F–J). Therefore TM and 4-PBA treatments were effective and successfully modeled ER stress.

Shear force is an intuitive and widely used index to measure the degree of postmortem muscle tenderness. The results of this study showed a decreasing trend in shear force with increasing postmortem ageing time in agreement with previous studies^[22]. Previous studies found that treatment of chicken breast with quercetin^[8], ultrasound^[18], and ultrasound combined with sodium bicarbonate^[11] reduced shear force and improved meat tenderness. The myofibril fragmentation index (MFI) reflects the integrity of myofibrils and their skeleton proteins. Higher MFI values indicate more severe damage to myofibrils, and MFI values are negatively correlated with shear force and positively correlated with meat tenderness^[16]. TM-induced ER stress significantly reduced shear force and increased MFI value (Figs. 2A and C), while inhibition of ER stress did the opposite (Figs. 2B and D), thus ER stress contributed to improved postmortem chicken tenderness. The increase of MFI value was associated with the breakage of myofibrils into small pieces in the I-band^[8], so the ultrastructure of chicken breast muscle was further observed by TEM. I-bands, A-bands, and M-lines all showed significant changes after TM treatment, with varying degrees of structural damage and severe degradation of myofibrils (Figs. 3G–I). M-lines lysis, A-bands shortening, I-bands enlargement, and myofibrils degradation were also observed in ultrasound-treated chicken breast^[17]. Therefore, the relative deformation and progressive blurring of Z-lines observed may be related to the localization of desmin. Desmin is a key myofibrillar protein, which has previously been shown to be located around Z-lines^[23], therefore desmin degradation may cause a weakening of Z-lines connection with adjacent myofibrils. Further investigation of desmin in chicken breast muscle treated with TM and 4-PBA found that ER stress aggravated desmin degradation (Figs. 4A–C). These results suggested that induction of ER stress promoted the degradation of myofibrillar protein desmin, further destroyed myofibrillar structure, and increased meat tenderness during postmortem ageing.

Endogenous proteases can degrade the cytoskeletal myofibrillar proteins, which is the main reason for the increased meat tenderness during postmortem. Caspase-12 is a specific apoptotic factor to ER stress, which is not expressed in any other apoptotic pathway^[19]. Tumor necrosis factor receptor-associated factor 2 (TRAF2) formed a stable complex with pro-caspase-12, upon ER stress, the activated IRE1 recruits TRAF2 and binds it to the ER membrane, so pro-caspase-12 would be released from TRAF2, and simultaneously promoted dimerization to generate active caspase-12 which drives apoptosis through the caspase cascade reaction^[24]. One study found significant expression of caspase family proteases during pork ageing and a significant increase in caspase-12 in semitendinosus^[25]. Therefore, the activation of caspase-12 during postmortem ageing in this study (Figs. 4D and E) might associate with the activation of IRE1 and indicated apoptosis caused by ER stress. The traditional theory of meat ageing suggests that calpain is the most important enzyme involved in the degradation of myofibrils during postmortem ageing, especially μ -calpain. It was found that μ -calpain was activated during postmortem ageing in chicken breast^[26] and lamb^[27], and the activation

of μ -calpain was positively correlated with the degradation of key structural proteins. The results suggested that ER stress degraded desmin and disrupted myofibrillar structure, which was caused by activating μ -calpain (Figs. 4D and F). In addition, the degradation of I-bands may be related to the role of μ -calpain, which was found to be predominantly localized in I-bands in bovine muscle. μ -Calpain interacted with titin to concentrate μ -calpain in the N-line region (the denser lateral stripe in I-bands), and protein hydrolysis was detected in this region during early postmortem^[28]. Previous studies have shown that apoptotic enzyme plays a synergistic role with μ -calpain in myofibrillar protein hydrolysis^[29]. Moreover, the degradation of myofibrillar proteins and structure of chicken breast meat by apoptosis inducer benefited from apoptotic cascade reaction^[30]. Therefore, ER stress promoted myofibrillar protein degradation probably due to the combined action of caspase-12 and μ -calpain, and related to apoptosis caused by ER stress.

Apoptosis refers to the genetically controlled, autonomous, and orderly death of cells to maintain the stability of the internal environment. When animals are slaughtered and bled, the nutrient and oxygen supply to myocytes are interrupted, which finally leads to cell death. The important role of apoptosis in ageing has been widely discussed in the theoretical research of meat with the introduction of apoptosis into the field of postmortem muscle tenderization, and is considered to be highly relevant to the postmortem muscle tenderization process^[16,31]. The Bcl-2 family is one of the most important regulators, containing both anti-apoptotic members and pro-apoptotic members, which can regulate mitochondrial outer membrane permeability and control the release of cytochrome c from mitochondria. Bax expression was significantly increased in postmortem ageing of beef, and cytochrome c was released from mitochondria to cytoplasm^[11]. CHOP, as a major apoptotic factor of ER stress, regulates several downstream apoptotic mechanisms. Bcl-2 family members are among the downstream targets of CHOP-induced apoptosis. Sustained activation of CHOP upregulates BH3-only protein Bim, which in turn induces the Bax-regulated mitochondrial apoptosis pathway^[32]. The results of our study were consistent with previous findings that TM-induced ER stress activated CHOP, inhibited anti-apoptotic member Bcl-xL, enhanced pro-apoptotic members Bim and Bax expression, altered mitochondrial membrane permeability, promoted cytochrome c release, and activated the mitochondrial apoptotic pathway (Figs. 5A–F), whereas inhibition of ER stress inhibited the mitochondrial apoptosis (Figs. 5G–L). Furthermore, IRE1, in addition to initiating splicing of *XBP1* mRNA, also degraded miR17 in the overactivated state, finally promoted Bax/Bak-dependent apoptotic signaling^[33–34]. Thus the effect of the Bcl-2 family may also be related to the activation of IRE1. It was found that cells and mice knocked out of both Bax and Bak lacked IRE1 signaling when undergoing ER stress and showed strong resistance to apoptosis, and stronger interaction between Bax and IRE1 was observed in wild-type cells^[35]. Therefore, ER stress might regulate the Bcl-2 family by activating CHOP and IRE1, thereby activating the mitochondrial apoptosis pathway during postmortem ageing and further promoting meat tenderization.

The current study though reveals that apoptosis is the main mode of myocyte death after slaughter. However, myocytes are typical multinucleated cells, their mode of death is more complex than that of single cells. In addition, the internal environment changes

are dramatic after slaughter. After the animal has been slaughtered and bled, on the one hand, it drives the myocytes towards death; on the other hand, the body instinctively activates its defense system, protective factors are activated, and cells undergo autophagy to protect the injured myocytes. Autophagy can protect cells by reducing ER stress through the degradation of unfolded/misfolded proteins^[36]. However, prolonged exposure of cells to ER stress induces autophagy-dependent cell death mechanisms that lead to cellular damage and upregulates the expression of pro-apoptotic molecules that ultimately cause apoptosis through a complex series of signaling pathways^[37]. To investigate whether autophagy occurs in postmortem ageing, the expression of several autophagic markers was examined. LC3 has two forms, and when autophagy occurs, the cytoplasmic form of LC3 (LC3I) is coupled to phosphatidylethanolamine to form LC3-phosphatidylethanolamine coupling (LC3II) and is recruited to the autophagosomes membrane. The LC3II/LC3I ratio is positively correlated with the number of autophagosomes and reflects the autophagic activity of cells^[38]. Beclin-1 is one of the important proteins in autophagy generation and regulates the formation of autophagic precursors. p62 is a selective autophagic bridging protein that binds to ubiquitinated proteins during autophagy and then translocates to autophagic lysosomes for degradation, therefore reduced p62 levels are often considered a sign of promoted autophagic activity^[39]. One study observed elevated beclin-1 protein and LC3II/LC3I expression in postmortem pork^[40]. Treatment of chicken muscle with quercetin also made LC3I transform to LC3II, increased beclin-1 expression, and induced autophagy^[8]. These were identical to the expression of autophagy in postmortem ageing induced by ER stress. In addition, down-regulation of p62 was also observed (Figs. 6A and D). Another study found that IRE1 activation by ER stress could regulate autophagy and autophagosome formation was inhibited in IRE1-deficient cells^[41]. Furthermore, XBP1 could bind to the promoter region of the beclin-1 gene to promote beclin-1 transcription and LC3II activation, thereby inducing autophagy^[42]. These may account for the ER stress regulated autophagy in the present study. Beclin-1 could regulate autophagy through its BH3 structural domain interacting with Bcl-xL. Moreover, the overexpression of beclin-1 promoted apoptosis, probably because beclin-1 inhibited Bcl-xL expression. Beclin-1 and Bax expression were positively correlated, and the two synergistically promote the conversion of autophagy to apoptosis^[43]. Thus excessive autophagy may mediate the onset of apoptosis through beclin-1. The obtained data showed that, ER stress caused autophagy and increased the expression of beclin-1 (Figs. 6A and B), which may further regulate the Bcl-2 family thereby inducing apoptosis. Class I PI3K activates mTOR through signal transduction, and mTOR is an autophagy-negative regulatory kinase. mTOR activation inhibits autophagy, and conversely, inhibition of mTOR induces autophagy to occur^[44]. Induction of ER stress decreased the expression of PI3K and mTOR and promoted autophagy (Figs. 6A, C and F), which was consistent with the above results. Diminished PI3K and mTOR expression were also found in quercetin-treated chicken breast muscle, which induced autophagy in postmortem ageing^[8]. Therefore, ER stress might induce autophagy by activating XBP1 and IRE1, further promoting autophagy by inhibiting the PI3K and mTOR. In addition ER stress increased the expression of beclin-1, which may promote autophagy towards apoptosis by interacting with the Bcl-2 family.

5. Conclusion

In conclusion, ER stress was effectively regulated by TM and 4-PBA through the IRE1 pathway and had a great effect on tenderness, apoptosis, and autophagy of chicken during postmortem ageing. ER stress degraded myofibrillar proteins by activating endogenous enzymes (caspase-12 and μ -calpain) to disrupt the myofibrillar structure, thereby reducing shear force, suggesting that ER stress played an active role in meat ageing and tenderization, effectively improving meat tenderness. Moreover, ER stress was able to active apoptosis by regulating the Bcl-2 family and promote autophagy. However, the detailed relationship between ER stress-induced autophagy and apoptosis remained to be investigated. This study illustrated the mechanism of meat ageing from the perspective of ER stress, which provided new ideas and insights for the regulation of meat tenderness.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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