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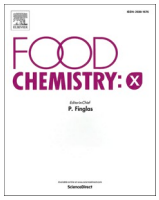

The correlation between Smac, IAPs and mitochondrial apoptosis, muscle tenderness during postmortem aging of Oula Tibetan sheep meat

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ABSTRACT

Oula Tibetan sheep meat has rich nutritional value but relatively poor tenderness. Recently, apoptosis of muscle cells has gradually become a research hotspot for improving meat tenderness during postmortem aging. Smac can promote the decrease of IAPs expression in tumor cells, thereby inducing mitochondrial apoptosis. However, the relationship between Smac, IAPs and mitochondrial apoptosis, muscle tenderness during postmortem meat aging is still unclear. Thus, the aim of this work was to explore the relationship between Smac, IAPs and mitochondrial apoptosis as well as muscle tenderness during postmortem meat aging. Smac concentration, IAPs concentration, pH value, ATP content, SDH activity, MPTP opening degree, MMP, caspase-3/9 activity, apoptotic rate, MFI and shear force value of Oula Tibetan sheep meat were measured at different aging times and correlation analysis was performed. Correlation analysis revealed that Smac, IAPs were markedly related to mitochondrial apoptosis and muscle tenderness during postmortem aging of Tibetan sheep meat. The results suggest that Smac may regulate IAPs to promote mitochondrial apoptosis and muscle tenderization in Oula Tibetan sheep meat during postmortem aging.

1. Introduction

Tibetan sheep are a unique grassland sheep breed in the Tibetan Plateau and its adjacent areas with about 40 million sheep (Shi et al., 2021). It has a strong ability to adapt to the environment of high cold and hypoxia, and has the advantages of rough feeding resistance, strong disease resistance and stable genetic traits. Oula Tibetan sheep has become an important economic and livelihood source for people in the Qinghai-Tibet Plateau and its adjacent areas due to their excellent wool quality and higher meat yield. Unfortunately, the tenderness of Oula Tibetan sheep meat is poor and affects edible quality (Shi et al., 2021). Tenderness is one of the important quality attributes, thus it is necessary to improve the tenderness of Oula Tibetan sheep meat. Postmortem aging is one of the important means to ameliorate meat tenderness. The improvement of meat tenderness during postmortem aging is mainly attributed to the hydrolysis of myofibrillar protein by endogenous enzymes, including cathepsins, calpains and caspases etc. (Sentandreu, Coulis, & Ouali, 2002). As researchers continue to explore the mechanism of muscle tenderization, apoptosis has gradually become the hot theory to explain meat tenderization during postmortem aging (Wang et al., 2018).

After the animal is slaughtered, the ischemic and hypoxic environment causes the supply of oxygen and nutrients in the body to be interrupted, which aggravates the anaerobic glycolysis reaction, causing the accumulation of lactic acid and the decrease of ATP content, thus creating a good condition for apoptosis (Chen et al., 2020). Currently, there are three pathways of apoptosis, including endogenous mitochondrial apoptosis pathway, endoplasmic reticulum stress apoptosis pathway and exogenous death receptor, the mitochondrial pathway is considered the principal apoptotic pathway (Yoshida et al., 1998). When mitochondrial membrane potential (MMP) decreases and mitochondrial permeability transition pore (MPTP) opening degree increases, pro-apoptotic factors (cytochrome *c* (Cyt—C), Smac, etc.) are released from mitochondria to cytoplasm and further induce mitochondrial apoptosis (Brenner & Mak, 2009; Wang et al., 2018).

In medical research, it has been confirmed that Smac (Smac is a second caspase activator released by mitochondria) and its mimics can act as inhibitors of IAPs (inhibitors of apoptosis proteins) to activate caspase and mediate apoptosis in cancer cells (Abhari et al., 2019). Many studies have shown that Smac may competitively bind to IAPs,

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making IAPs lose the ability to bind to caspases, thereby further increasing caspase activity and triggering apoptosis (Dietz et al., 2023; Fulda, 2017). In addition, Tian (Tian, Ji, & Zhang, 2021) also demonstrated that Smac inhibited XIAP expression by binding to the structural domains BIR3 or BIR2 of XIAP, thereby inducing apoptosis of cancer cells. On the other hand, some scholars have confirmed that endogenous Smac and its mimics can promote ubiquitination of IAPs, thereby entering the ubiquitin-proteasome pathway for degradation and promoting apoptosis (MacFarlane, Merrison, Bratton, & Cohen, 2002). Presently, eight IAPs (including NIAP, CIAP1, CIAP2, XIAP, Survivin, Bruce, ML-IAP, and ILP2) have been identified in medicine. Among them, the relationship between XIAP, CIAP1, CIAP2 and caspase has been extensively studied in the field of medical oncology (Fulda, 2017). In mammals, XIAP, CIAP1 and CIAP2 can inhibit caspase-9 activity by binding to pro-caspase-9, and can also inhibit the activation of caspase-3 by binding to caspase-3, however, the inhibitory effect of CIAP1 and CIAP2 on apoptosis is much weaker than that of XIAP (Dietz et al., 2023; Hunkeler, Jin, & Fischer, 2023; Vaux & Silke, 2003). In conclusion, studies have found that endogenous Smac and Smac mimics play a pro-apoptotic role in tumor cells by binding to IAPs and inhibiting their expression or promoting ubiquitination degradation of IAPs. However, it is not clear whether Smac and IAPs affect mitochondrial apoptosis and ameliorate muscle tenderness during postmortem meat aging. Therefore, the aim of this work was to explore the relationship between Smac, IAPs and mitochondrial apoptosis as well as meat tenderness during postmortem aging of Tibetan sheep meat, and to provide new insights for improving the theory of tenderization during postmortem aging of meat.

2. Material and methods

2.1. Animals and sample preparation

All procedures and protocols involving animals were approved by the Institutional Animal Care and Use Committee of Gansu Agricultural University (approved ID: GSAU-Eth-FSE-2024-003).

Twelve healthy, average age 4-year-old female Oula Tibetan sheep were executed by halal slaughter at Gansu Ando Halal Green Food Co., Ltd. This process was strictly carried out in accordance with national slaughter standards. After bleeding, the longissimus dorsi of the Tibetan sheep was quickly removed. The longissimus dorsi surface fat and connective tissue were eliminated and cut into pieces with an average weight of 60 g. Some meat samples were quickly frozen with liquid nitrogen as samples of 0 h (< 10 min). The other meat samples were aged at 4 °C for 6, 12, 24, 72, 120, and 168 h. At the end of each storage point, the pH and shear force of meat samples were determined, and the rest meat samples were vacuum-packed and stored at −80 °C to measure other indicators.

2.2. Measurement of ATP content

ATP content was assessed using the method of kit (Jian cheng Bioengineering Research Institute Nanjing, China). 1 g meat samples were mixed with 9 mL double distilled water and homogenized. Subsequently, homogenates were centrifuged at 3500 ×*g* for 10 min. Next, the mixture was placed in a boiling water bath for 10 min and then oscillated for 1 min. The mixed solution was centrifuged at 3500 ×*g* for 10 min and supernatant was taken for testing. 30 μL suspension was mixed with kit reaction reagents I (100 μL), II (200 μL), III(30 μL) and incubated for 30 min at 37 °C. Then 50 μL reagent IV was mixed with the mixture and centrifuged at 4000 ×*g* for 5 min. Subsequently, 300 μL supernatant and 500 μL reagent V were mixed and placed for 2 min. 500 μL reagent VI was added to the mixed solution, and the absorbance was measured by UV spectrophotometer (UV2550, Shimadzu Corp, Kyoto, Japan) at 636 nm. In addition, the blank tube, standard tube and contrast tube are set according to the instructions. Finally, ATP content was calculated according to the formula provided by the kit.

2.3. Measurement of pH

The pH value was measured by a portable pH meter (pHTesto-205, Testo Instruments International Trading Co., Shanghai, China) according to the method of Chen et al. (Chen et al., 2022). Firstly, the pH meter was calibrated with a buffer solution of pH 6.86 and pH 4.01. Subsequently, the bloodstain on the meat samples was rinsed with distilled water, and the residual water on the surface was dried with clean filter paper. The pH meter probe was randomly inserted into three different locations of meat samples and the average value was calculated after the readings stabilized.

2.4. Mitochondrial extraction

Mitochondrial extraction was performed using the previous study with minor modifications (Zhang, Li, Yu, Han, & Ma, 2019). 10 g meat samples were added to 100 mL mitochondrial isolation solution (0.005 mol/L 3- N-morpholine propane sulfonic acid, 0.7 mol/L sucrose, 0.5 % bovine serum albumin, 0.2 mol/L mannitol and 0.002 mol/L EDTA, pH 7.4), which were homogenized at 10,000 ×g for 1 min, then centrifuged at 1000 ×*g* for 10 min at 4 °C and the supernatant was discarded. Under the same centrifugation conditions, the supernatants were centrifuged then the supernatants were again centrifuged at 8000 ×*g* for 20 min at 4 °C to obtain mitochondria and the cytoplasmic.

2.5. Detection of MPTP opening degree

The absorbance was determined by the method of Wang et al. to indicate the degree of MPTP opening (Wang et al., 2018). 5 mL buffer (0.01 mol/L Tris-HCl, 0.25 mol/L sucrose, pH 7.4) was added to the mitochondrial precipitate to resuspend mitochondria, and the protein concentration of suspension was detected by the biuret method. Subsequently, the mitochondrial suspension concentration was adjusted to 0.3 mg/mL with MPTP test medium (0.23 mol/L mannitol, 0.003 mol/L Hepes, and 0.07 mol/L sucrose, pH 7.4). Next, 3 mL MPTP test medium was mixed with 1 mL mitochondrial solution and added to the cuvettes. The absorbance was determined at 540 nm with a UV spectrophotometer (UV2550, Shimadzu Corp, Kyoto, Japan).

2.6. MMP measurement

The determination of MMP changes was referred to a previous study (Wang et al., 2018). Firstly, the JC-1 staining working solution was configured according to the method of Wang et al. (2018). Subsequently, 0.1 mL mitochondrial suspension (0.1 mg/mL) was mixed with 0.9 mL JC-1 staining working solution (5-fold dilution) and incubated for 20 min at 37 °C. Finally, the fluorescence intensity value was determined by fluorescence spectrophotometer (RF 5301-PC Fluorescence Spectrophotometer, Shimadzu Corp, Kyoto, Japan). The ratio of red and green fluorescence intensity reflected the level of mitochondrial membrane potential (When detecting JC-1 monomer: Ex/Em: 490/525 nm; When detecting JC-1 polymers: Ex/Em: 530/590 nm).

2.7. Determination of mitochondrial succinate dehydrogenase (SDH) activity

According to the instructions of the kit (Jian cheng Bioengineering Research Institute Nanjing, China) to detect SDH activity of mitochondria. The working solutions were configured according to instructions of kit. 100 μL mitochondrial suspension was mixed with the working solution. The mixed solution was added to the colorimetric cup. Subsequently, absorbance was measured by UV spectrophotometer at 600 nm for 5 s and 65 s, respectively. Finally, SDH activity was calculated according to formula in the kit.

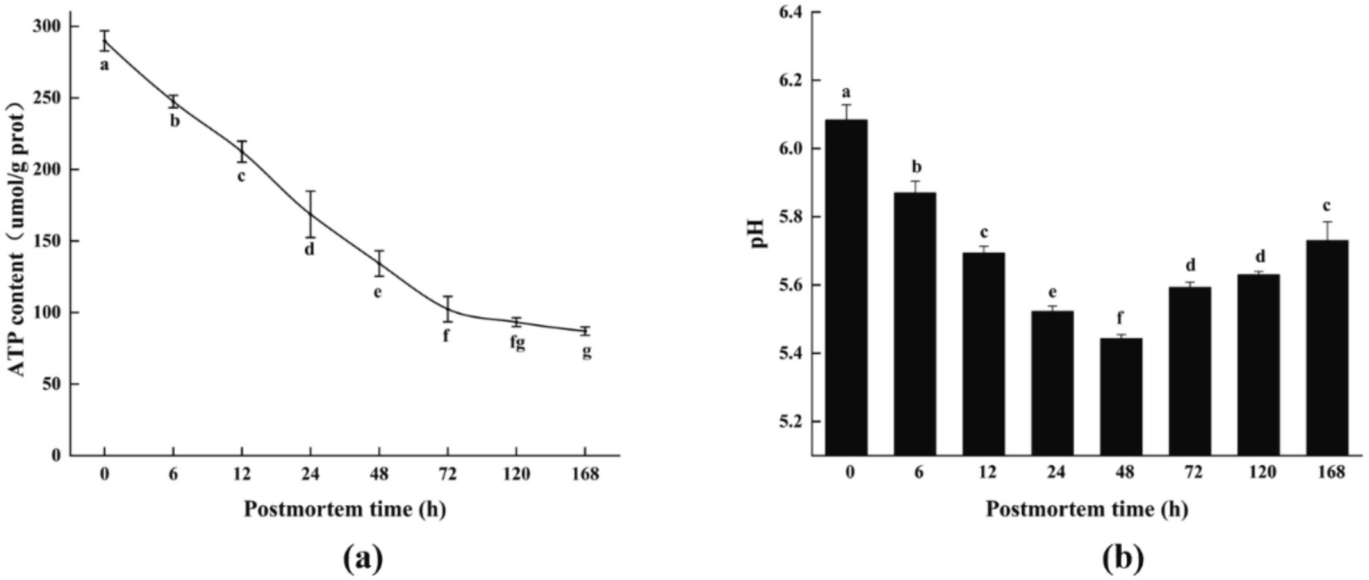

Fig. 1. Changes in the intramuscular environment during postmortem aging of Oula Tibetan sheep meat. Changes in ATP content during postmortem aging of Tibetan sheep meat (a). Changes in pH during postmortem aging of Tibetan sheep meat (b). a – g, different letters indicate significant differences between aging times *($P < 0.05$, $n = 8$)*.

2.8. Determination of cytoplasm Smac and IAPs concentration

The concentration of Smac was determined by the method of the kit (Guduo Biotechnology Co., Shanghai, China). 10 μL cytoplasm solution was mixed with 40 μL sample diluent. The mixture was incubated on an ELISA plate with 100 μL HRP-Conjugate reagent at 37 °C for 1 h. Next, the incubated liquid was discarded and the ELISA plate was washed. Subsequently, 50 μL chromogen Solution A and B were added to the ELISA plate and incubated in darkness at 37 °C for 15 min. Finally, the incubation solution was mixed with the 50 μL stop solution. The mixed solution absorbance was immediately measured at 450 nm using a microplate reader (Spectramax, MD, USA). The concentration of cytoplasmic Smac protein was calculated according to the absorbance (based on standard curve).

The concentration of IAPs were assessed using the method of kit (Guduo Biotechnology Co., Shanghai, China) with slight modification. 2 g meat samples were mixed with 18 mL normal saline and homogenized at 15000 ×g for 1 min. Next, the homogenates were centrifuged at 3000 ×*g* for 10 min. Subsequently, the supernatant was collected for IAPs concentration detection. 10 μL supernatant was mixed with 40 μL sample diluent. The mixture was incubated on an ELISA plate with 100 μL HRP-Conjugate reagent at 37 °C for 1 h. Next, the incubated liquid was discarded and the ELISA plate was washed. Subsequently, 50 μL chromogen Solution A and B were added to the ELISA plate and incubated in darkness at 37 °C for 15 min. Finally, the incubation solution was mixed with the 50 μL stop solution and the absorbance was immediately measured at 450 nm using a microplate reader. The concentration of IAPs was calculated according to the absorbance (based on standard curve).

2.9. Caspase activity assay

The activity of caspase was determined by the corresponding method of kit (Beijing Solarbio Science & Technology Co, Ltd., China). 0.1 g minced meat samples were mixed with 1 mL reagent II and centrifuged at 12000 ×*g* for 10 min. Subsequently, 50 μL supernatant was mixed with the reaction reagent and incubated in the microplate at 37 °C for 1 h. Finally, the absorbance of the incubation solution was determined at 405 nm by a microplate reader. The protein concentration of the supernatant was calculated using Bradford method. Caspase activity was calculated based on the formula of kit.

*2.10. Rate of **apoptosis***

The apoptotic nucleus was detected by the TdT-mediated dUTP nick end labeling method (TUNEL) assay kit (Roche Group, Switzerland). Paraffin sections of 10 μm were prepared according to the method of Hou et al. (Hou, Liu, Tian, & Zhang, 2020). After dewaxing, the sections were sealed with methanol solution and 3 % H_2O_2 for 10 min at 37 °C. Subsequently, the sections were incubated with 100 μL proteinase K for 30 min at 37 °C. Then, the sections were washed three times with PBS for 5 min each time. Next, The sections were incubated with 50 μL TUNEL reaction solution (A: B = 1: 30) in the dark for 60 min at 37 °C. The negative control group was treated with 50 μL B solution instead of TUNEL reaction solution. The positive control group was incubated with 100 μL DNase I for 10 min at room temperature before adding TUNEL reaction solution. The treated tissue sections were blocked with glycerol and observed under digital slice scanner (Pannoramic 250, 3DHISTECH, Hungary). Each sample was counted at least three times, and the apoptosis rate was expressed as the ratio of apoptotic nucleus to normal nucleus.

2.11. Determination of MFI and shear force

The MFI of meat samples was determined at different aging time points (Shi et al., 2021). First, 2 g meat samples were homogenized at 12000 ×g for 1 min with 18 mL MFI buffer (0.01 mol/L KCl, 0.02 mol/L K_3PO_4, 1 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1 mmol/L NaN_3, pH 7.1). The homogenates were centrifuged at 1000 ×*g* for 15 min at 4 °C. Next, the 18 mL buffer solution was added to the precipitate again and centrifuged. Finally, the 10 mL buffer was fully mixed with the precipitate and the myofibrillar protein solution was immediately collected through a nylon mesh sieve (200 mesh). The concentration of myofibrillar protein solution was adjusted to 0.5 mg/mL with MFI buffer. The absorbance was detected at 540 nm with a spectrophotometer. This absorbance is multiplied by 200 to obtain MFI.

The shear force was determined as reported by Wang et al. (2018) with some modifications. The meat samples were placed in a cooking bag and heated water bath at 80 °C. When the central temperature of the meat samples reaches 75 °C, remove and cool to room temperature.

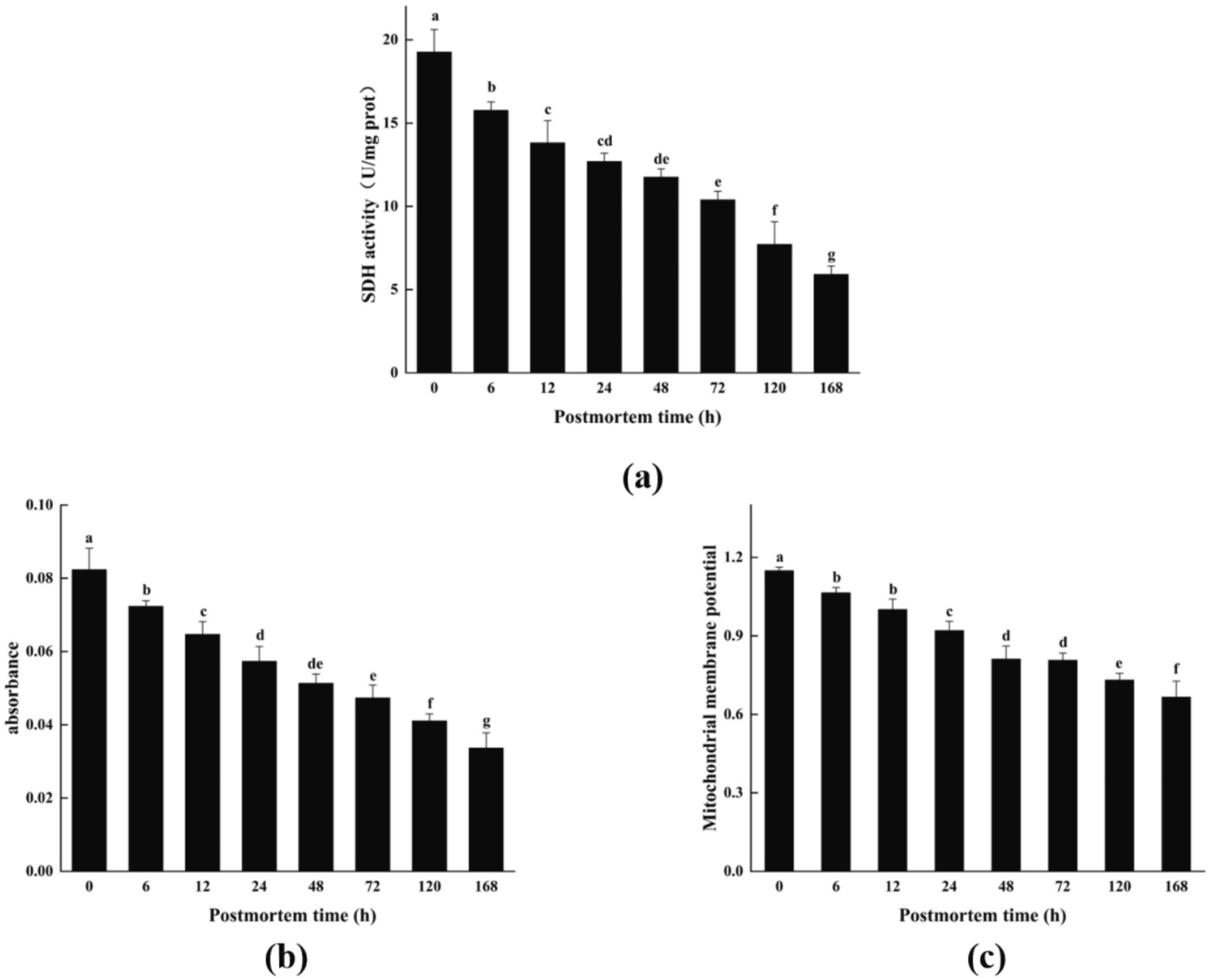

Fig. 2. Changes in mitochondrial damage indices during postmortem aging of Oula Tibetan sheep meat. Changes in SDH activity during postmortem aging of Tibetan sheep meat (a). Changes in MPTP opening degree during postmortem aging of Tibetan sheep meat (b). Changes in mitochondrial membrane potential during postmortem aging of Tibetan sheep meat (c). a – g, different letters indicate significant differences between aging times *($P < 0.05$, $n = 8$)*.

Subsequently, the samples were cut along the muscle fiber direction with a columnar sampler (Φ 1.27 cm). The meat column was placed vertically on the V-shaped tool holder of the tenderometer (C-LM4, Harbin, China) for shearing and recording the value.

2.12. Statistical analysis

All data were measured three times. Data calculated means and standard deviations (± SD) using Microsoft Office Excel 2016. One-way analysis of variance (ANOVA) and multiple comparisons (Duncan) in IBM SPSS 26.0 software were used to analyze the significance of the data. Correlation analysis was performed using SPSS 26.0 (Pearson method). Origin 2021 software was used for plotting.

3. Results and discussion

3.1. Changes in the ATP content and pH value

ATP is an important index to reflect the energy level of cells (Lokanath et al., 2005). Muscle cells are prone to ischemia and hypoxia in the early stages of postmortem aging. To maintain metabolism, muscle cells rely on the anaerobic glycolytic pathway to produce ATP to meet energy needs (Zhang et al., 2019). The occurrence of anaerobic glycolysis in muscle cells leads to lactic acid accumulation and pH decline (Zhang, Liu, et al., 2019).

ATP content decreased significantly in Tibetan sheep meat during postmortem aging, especially at 0–72 h *($P < 0.05$)* in Fig. 1-(a). The pH value decreased markedly at 0–48 h *($P < 0.05$)*, reaching a minimum value of 5.44, and then increased markedly (48–168 h) in Fig. 1-(b). There are two reasons for the decline of ATP content in postmortem Tibetan sheep meat. On the one hand, postmortem muscle cell lacks oxygen and occurs anaerobic glycolysis, thereby reducing ATP synthesis (Lokanath et al., 2005). On the other hand, ATP is hydrolyzed during postmortem muscle aging. The pH value decreased in the early postmortem stages, which was attributed to ATP hydrolysis and lactic acid accumulation. While pH value increased from 48 to 168 h, which might be due to alkaline substances of protein breakdown, for instance amines and ammonia, etc. (Shi et al., 2021). The similar results were reported by Zhang et al. (Zhang, Liu, et al., 2019) who found that the ATP content and pH value of pork markedly decreased in the early postmortem stages *($P < 0.05$)*. Furthermore, many scholars believed that ATP and pH value were closely related to apoptosis (Eguchi, Srinivasan, Tomaselli,

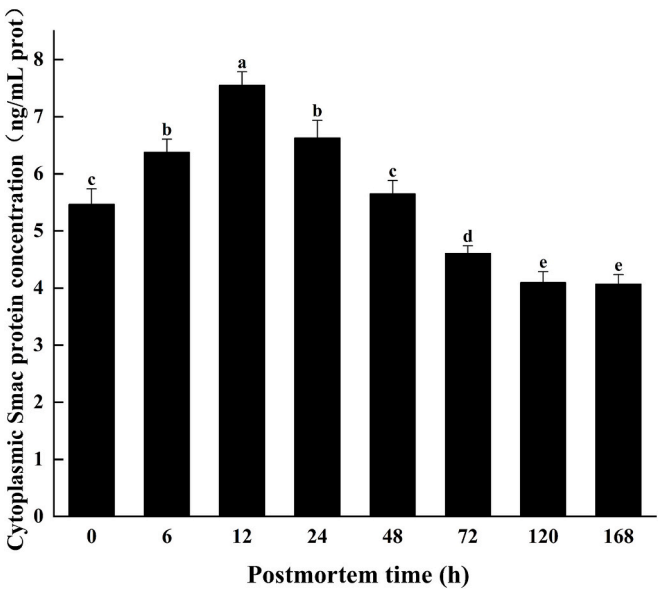

Fig. 3. Changes in concentration of cytoplasmic Smac during postmortem aging of Oula Tibetan sheep meat. a – e, different letters indicate significant differences between aging times *(P < 0.05, n = 8)*.

Shimizu, & Tsujimoto, 1999; Sergeeva et al., 2017). In summary, the pH value and ATP content of Tibetan sheep meat markedly decreased in early postmortem aging, which may provide favorable conditions for the occurrence of muscle apoptosis.

3.2. Changes in mitochondrial damage indices

SDH can participate in the tricarboxylic acid cycle (TCA) and effectively reflect mitochondrial function. It is one of the important markers of mitochondrial damage (Lorendeau et al., 2017). The SDH activity in Oula Tibetan sheep meat during postmortem aging was investigated (Fig. 2-a). The results showed that SDH activity obviously declined with the prolongation of aging time *(P < 0.05)*. The similar results were reported by some researchers who found that SDH activity decreased with the decline of ATP content (Wang et al., 2023). The decline of SDH activity might be ascribed to the decrease of ATP content during postmortem aging of Tibetan sheep meat, which severely disrupted the TCA cycle. In conclusion, the mitochondrial SDH activity in Oula Tibetan sheep meat decreased with the extension of aging time.

The low permeability opening of MPTP is very important for maintaining cellular homeostasis. When cells are stimulated, the high permeability opening of MPTP can cause changes in MMP, further impairing mitochondrial function (Wang et al., 2018). Fig. 2-(b) demonstrated the change in the MPTP opening degree. During postmortem aging, the absorbance showed a significant decrease trend, which meant that the MPTP opening degree had a significantly increased trend *(P < 0.05)*. At 168 h, the MPTP opening degree was significantly increased by 59.05 % compared to postmortem 0 h.

As shown in Fig. 2-(c), MMP showed an overall significant decline trend during postmortem aging *(P < 0.05)*. At 168 h, the MMP was 42.02 % less than at early postmortem aging (0 h) *(P < 0.05)*. The results were in agreements with Zhang, Li, et al. (2019), who reported that the MMP in bovine muscle was significantly decreased from 6 to 72 h, while the degree of MPTP opening degree was greatly increased *(P < 0.05)*. The decrease of MMP and increase of MPTP opening degree may be due to changes in the intramuscular environment (Murphy, 2009). Additionally, Chen et al. (2020) also found that the openness of MPTP increased with the extension of aging time, especially in the period of pH and ATP significant decrease, which is similar to the results of the present study. Therefore, based on the above research results, we believe that the decrease of ATP content and pH value may induce mitochondrial damage during postmortem aging of Tibetan sheep meat.

3.3. Changes in concentration of cytoplasmic Smac

Smac is a pro-apoptotic protein released by mitochondria. It may increase caspase activity by inhibiting the binding of IAPs and caspase (Dietz et al., 2023).

Fig. 3 demonstrated that Smac concentration increased significantly from 0 to 12 h *(P < 0.05)*, then began to decline markedly *(P < 0.05)*. The increase of Smac concentration at 0–12 h may be due to the enhancement of MPTP opening degree, which further induces the release of Smac from mitochondria into cytoplasm (Hu et al., 2012; Wang et al., 2018). Smac concentration declined in the late postmortem period probably because XIAP promoted the ubiquitination degradation of Smac (MacFarlane et al., 2002), In addition, smac bound to IAPs, which could also cause the decline of Smac concentration. In summary, Smac is released from mitochondria and may promote the occurrence of mitochondrial apoptosis.

3.4. Changes in concentration of IAPs

IAPs are endogenous inhibitors of apoptosis. Previous studies reported that XIAP, CIAP1 and CIAP2 could inhibit caspase-3 activity by binding to caspase-3, and could also bind to pro-caspase-9 further inhibited the activation of caspase-9 (Dietz et al., 2023; Hunkeler et al., 2023).

As shown in Fig. 4-(a), the concentration of XIAP overall markedly decreased during postmortem aging. At 168 h, the concentration of XIAP was 61.39 % less than at 0 h *(P < 0.05)*. Fig. 4-(b) showed the concentration of CIAP1 decreased substantially during postmortem aging *(P < 0.05)*. At 168 h, the concentration of CIAP1 was 45.93 % less than at 0 h *(P < 0.05)*. CIAP2 concentration increased from 0 to 12 h, then declined markedly (Fig. 4-(c)). These findings were similar to the research results of Zou et al. (2023) who found that the expression of XIAP gene at 12 h was lower than at 1 h during postmortem aging of beef (Zou et al., 2023). Additionally, Bagnjuk et al. (2019) demonstrated that Smac could block the proliferation of IAPs in ovary cells, reducing the level of IAPs expression, and ultimately induced apoptosis (Bagnjuk et al., 2019). The decrease of XIAP concentration during postmortem of muscle aging may be related to Smac binding to XIAP or XIAP degradation via ubiquitin-proteasome pathway (García-Gutiérrez, Fallahi, Aboud, Quinn, & Matallanas, 2022; Mamriev et al., 2020). The reduction of CIAP1 and CIAP2 concentrations during postmortem aging of Tibetan sheep meat was possibly attributed to their degradation by ubiquitin-proteasome pathway (Conze et al., 2005; Sekine et al., 2008). In sum, the concentrations of XIAP, CIAP1 and CIAP2 in Tibetan sheep meat overall markedly decreased with the prolongation of aging time.

3.5. Changes in caspase activity

Caspase-9 and caspase-3 play important roles in mitochondrial apoptosis during postmortem aging of muscle (Hou, Zhu, Lu, & Zhang, 2021; Wang et al., 2018).

Caspase-9 activity increased significantly from 0 to 6 h *(P < 0.05)*, then decreased markedly (Fig. 5-(a)). Caspase-3 activity increased significantly at 0–12 h *(P < 0.05)*, then decreased markedly (Fig. 5-(b)). The similar result was reported by Zhang et al. (Zhang, Ma, & Kim, 2020) who found that caspase-3 activity significantly increased in pigs PM at 2–24 h and declined significantly at 24–48 h *(P < 0.05)*. This study found that caspase-9 activation preceded caspase-3 during postmortem aging, which was consistent with the report by Zhang et al. (Zhang, Li, et al., 2019). In the early stages of postmortem muscle aging, the increase of caspase-9 activity may be attributed to the effect of pro-apoptotic Smac, which leads to a decrease of the inhibitory effect of XIAP on caspase-9 (Shiozaki et al., 2003). There are some reasons for the

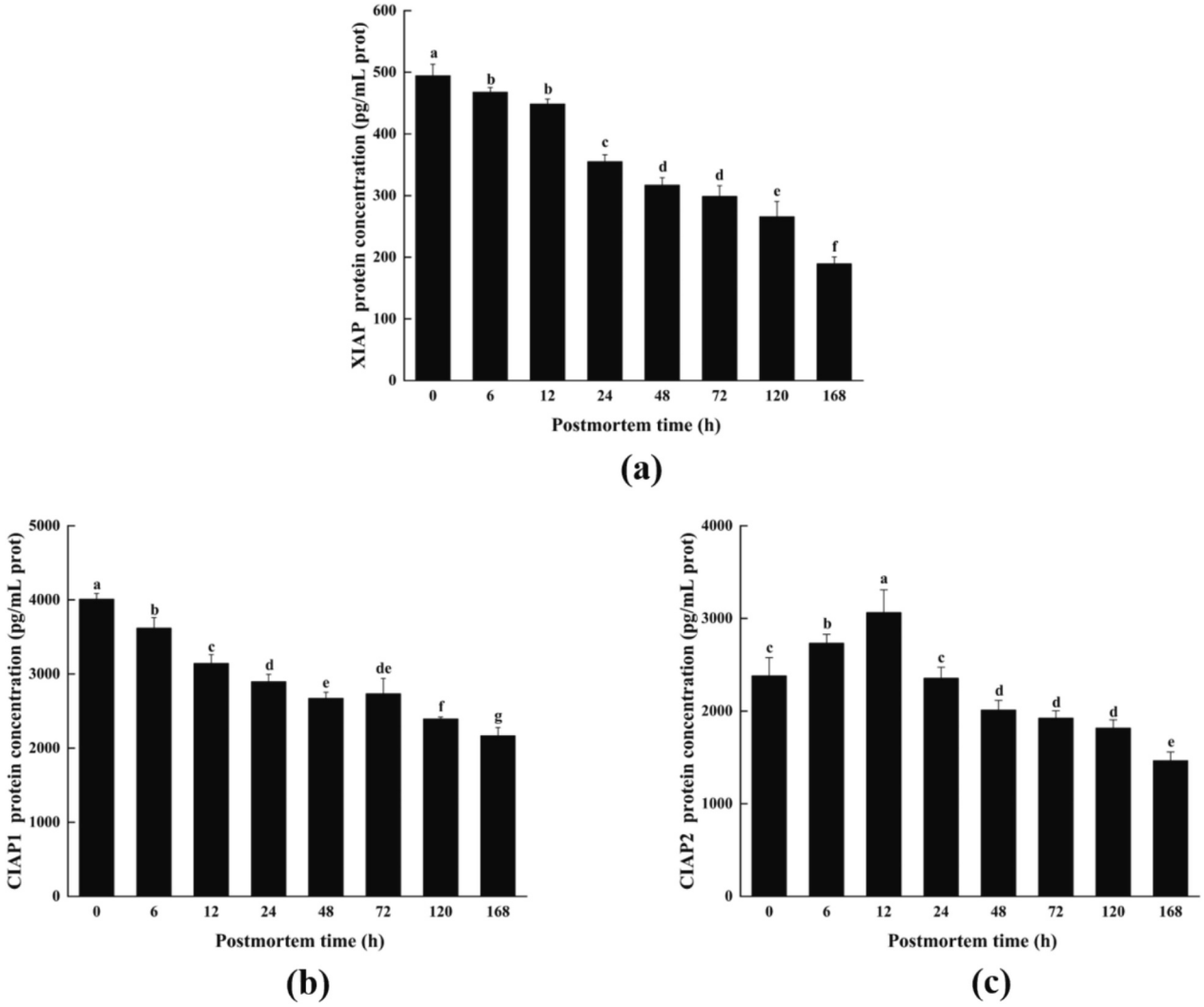

Fig. 4. Changes in concentration of IAPs in postmortem aging of Oula Tibetan sheep meat. Changes in XIAP concentration during postmortem aging of Tibetan sheep meat (a). Changes in CIAP1 concentration during postmortem aging of Tibetan sheep meat (b). Changes in CIAP2 concentration during postmortem aging of Tibetan sheep meat (c). a – g, different letters indicate significant differences between aging times *($P < 0.05$, $n = 8$)*.

decrease of caspase-9 activity in the late stages of postmortem aging. Firstly, the decrease of caspase-9 activity may be due to a decrease of Smac concentration, which causes the enhancement of the inhibitory effect of IAPs on caspase-9 (Jiang, Xiao, Shi, Liu, & Xiao, 2005). Secondly, caspase-9 may be involved in the formation of apoptotic vesicles on the mitochondrial pathway (Wang et al., 2018), which is further depleted. Lastly, caspase-9 may also be involved in other biochemical reactions in the cell, resulting in caspase-9 activity decline. The pro-apoptotic effect of Smac may increase the activity of caspase-9 and further activate capase-3. In conclusion, this study speculated that Smac may activate caspase-9 and caspase-3 by interacting with IAPs during postmortem aging of Tibetan sheep meat.

3.6. Changes in the rate of apoptosis

The representative TUNEL photographs of Oula Tibetan sheep LL were shown in Fig. 6-(a). The nuclei of normal cell are bluish, and the nuclei of apoptotic cell are greenish and overlap with blue light (Fig. 6-(a)). We found that the nuclei of apoptotic cell gradually increased in Tibetan sheep meat with the extension of aging time (Fig. 6-(a)).

The apoptosis rate of Oula Tibetan sheep meat in Fig. 6-(b) has been demonstrated. From Fig. 6-(b), we knew that the apoptosis rate of muscle cells in Oula Tibetan sheep overall markedly increased during postmortem aging *($P < 0.05$)*. It increased substantially with 96.33 % at 168 h compared to 0 h apoptosis rate *($P < 0.05$)*. The similar result was reported by Zhang et al. (Zhang et al., 2013) who found that the number of apoptotic cell nuclei dramatically increased in duck skeletal muscle during postmortem aging *($P < 0.05$)*. Based on the above findings, we believed that the increase of apoptosis rate during postmortem aging of Oula Tibetan sheep meat might be due to the activation of caspase-9 and caspase-3 by smac, which induce mitochondrial apoptosis. In short, the apoptosis rate of Tibetan sheep meat overall markedly increased with the prolongation of aging time.

3.7. Changes in MFI and shear force value

Tenderness of meat is an important quality attribute that affects consumer satisfaction. The shear force value can reflect meat tenderness, the smaller the shear force, the better the meat tenderness. MFI showed the degree of myofibrillar proteolysis. Postmortem meat tenderization is principally attributed to the degradation of myofibrillar protein (Shi et al., 2021).

As shown in Fig. 7-(a), the shear force value obviously increased at 0–48 h and then substantially declined *($P < 0.05$)*. Fig. 7-(b) illustrated the change in MFI during postmortem aging of Oula Tibetan sheep meat. The MFI overall markedly increased from 0 to 168 h *($P < 0.05$)*. The

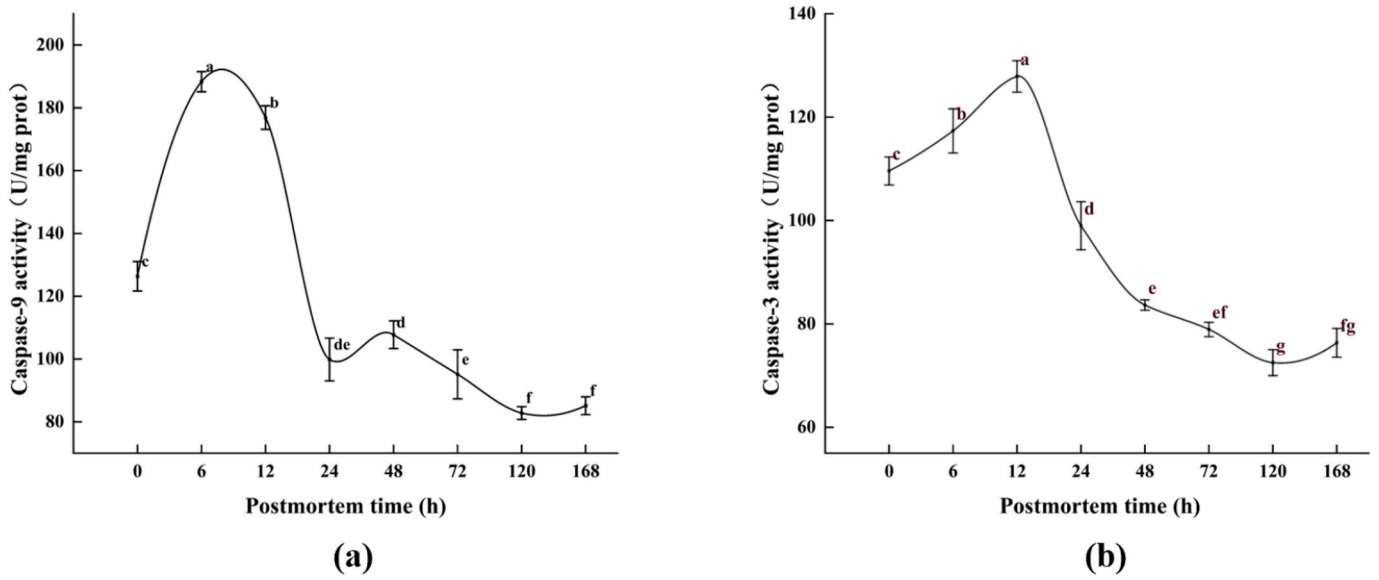

Fig. 5. Changes in caspase-9 (a) and caspase-3 (b) activity during postmortem aging of Oula Tibetan sheep meat. a – g, different letters indicate significant differences between aging times *(P < 0.05, n = 8)*.

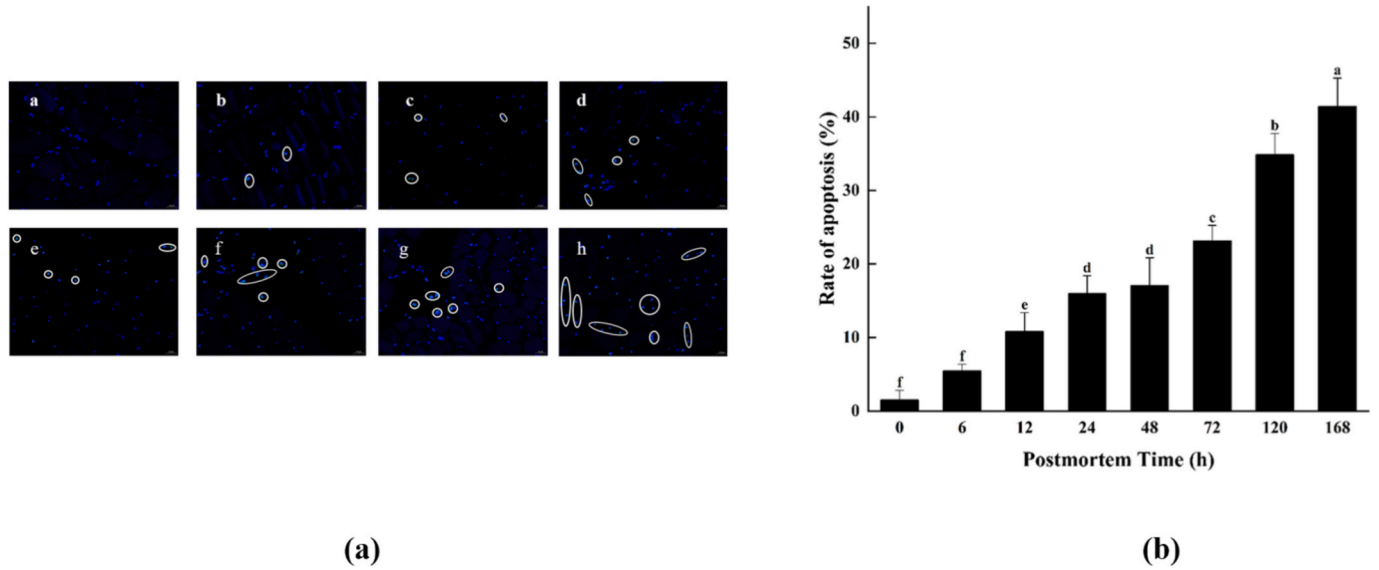

Fig. 6. TUNEL photographs of nuclei during postmortem aging of Oula Tibetan sheep meat (a-h). a-h represents 0, 6, 12, 24, 48, 72, 120, and 168 h postmortem aging of Oula Tibetan sheep meat (Fig. 6-(a)). Changes in the rate of apoptosis during postmortem aging of Oula Tibetan sheep meat (Fig. 6-(b)). a–f, different letters indicate significant differences between aging times *(P < 0.05, n = 8)*.

above findings were similar to those of Wang et al. (2018) who reported that the shear force of yak meat increased significantly from 0 to 72 h, then gradually decreased during postmortem aging. The increase of shear force value at 0–48 h may be due to the rapid decrease of pH during postmortem aging, which causes muscle rigidity (Zhang, Liu, et al., 2019). In addition, Shi et al. (2021) found that MFI increased significantly during postmortem aging of Oula Tibetan sheep meat *(P < 0.05)*, which was consistent with the study. The degradation of myofibrillar protein by endogenous enzymes in the late stages of postmortem aging led to a decrease of the shear force value and an improvement of meat tenderness (Huang, Huang, Xue, Xu, & Zhou, 2011). Endogenous enzymes include caspases, calpains, cathepsins and so on. Hence, we speculate that the improvement of Tibetan sheep meat tenderness during postmortem aging may be attributed to activation of caspase-3, which degrades myofibrillar fibrillar proteins.

3.8. The correlation between Smac, IAPs and mitochondrial apoptosis, muscle tenderness of Oula Tibetan sheep meat during postmortem aging

As shown in Fig. 8, we found that cytoplasmic Smac concentration was positively associated with caspase-9 activity and caspase-3 activity *(P < 0.05)* during postmortem aging of Tibetan sheep meat. The concentration of IAPs were positively correlated with mitochondrial damage indexes, and negatively correlated with apoptosis rate and MFI *(P < 0.01)*. In addition, cytoplasmic Smac concentration was positively associated with apoptosis rate, MFI and negatively correlated with mitochondrial damage indexes *(P < 0.05)* in the early stages of postmortem aging of Tibetan sheep meat. IAPs concentration were negatively correlated with caspase-9/3 activity in the early stages of postmortem aging of Tibetan sheep meat *(P < 0.05)*.

The results of this study were partially similar to those reported by

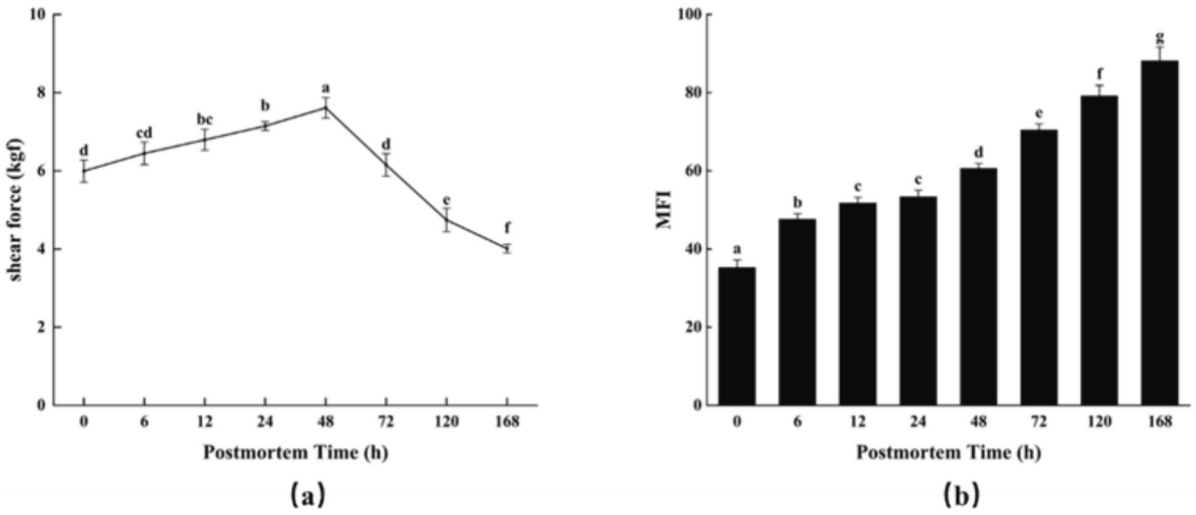

Fig. 7. Changes in shear force (a) and MFI (b) during postmortem aging of Oula Tibetan sheep meat. a – g, different letters indicate significant differences between aging times *(P < 0.05, n = 8)*.

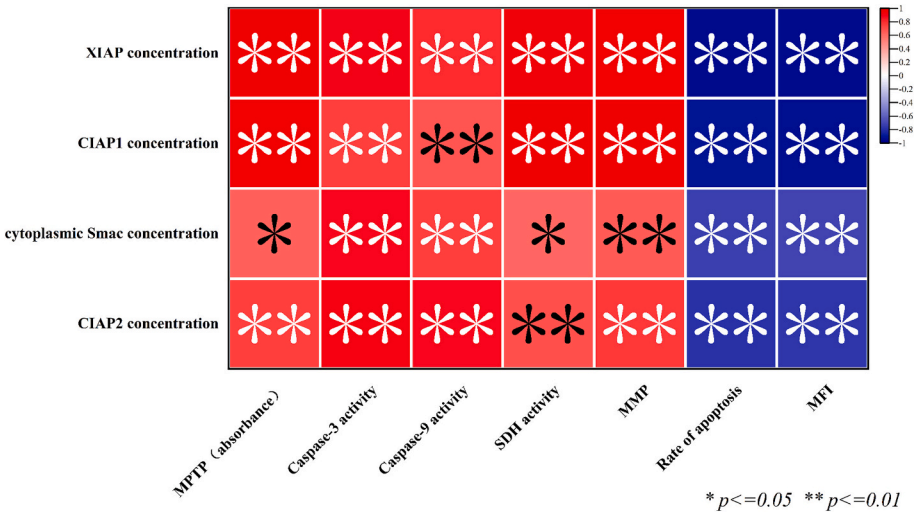

Fig. 8. The correlation between Smac, IAPs and mitochondrial apoptosis, muscle tenderness of Oula Tibetan sheep meat during postmortem aging. * express a significant *(P < 0.05)*, ** indicates highly significant *(P < 0.01)*.

De Oliveira Lima et al. (2009) who reported that cytoplasmic Smac immunoexpression in tumor cells was correlated with immunoexpression of CIAPs, XIAP and caspase-3, and that CIAPs immunoexpression was also correlated with immunoexpression of caspase-3 *(P < 0.05)*. Additionally, previous studies found that Smac acted on IAPs to trigger the specific apoptosis cascade reaction (Kitada, Yamaguchi, Rassenti, Kipps, & Reed, 2009). After animal slaughter, ischemia and hypoxia lead to the interruption of the supply of oxygen and nutrients in muscle cells. The occurrence of anaerobic glycolysis in muscle cells leads to lactic acid accumulation and the decrease of ATP content (Chen et al., 2020). The decrease of pH and ATP may cause mitochondrial damage in muscle cells and further lead to the release of apoptotic factors such as Smac from mitochondria to cytoplasm (Brenner & Mak, 2009). Smac may bind to IAPs or promote ubiquitination degradation of IAPs, thereby amplifying the caspase cascade reaction (Fulda, 2017). Activation of caspase-9 and caspase-3 may induce mitochondrial apoptosis and degrade myofibrillar proteins, which ultimately improve meat tenderness (Huang et al., 2016). The result may be attributed to the release of Smac from mitochondria into cytoplasm, which relieves the inhibitory effect of IAPs on caspases by interacting with IAPs, thereby activating caspase-3. In addition, The IAPs were positively correlated with mitochondrial damage indexes, and negatively correlated with apoptosis rate and MFI, whish is possibly because IAPs were inhibited, thereby causing intensification of apoptosis. Therefore, Smac may interact with IAPs during postmortem aging of Tibetan sheep meat and promote mitochondrial apoptosis as well as contribute to ameliorate meat tenderness. Nevertheless, the exact mechanism remains to be further explored and confirmed.

4. Conclusion

In this study, the cytoplasmic Smac concentration in Oula Tibetan sheep meat increased significantly from 0 to 12 h, then began to decline markedly. Three IAPs concentrations in Oula Tibetan sheep meat showed an overall significant downward trend. Correlation analysis revealed that Smac, IAPs were markedly related to mitochondrial apoptosis and muscle tenderness during postmortem aging of Tibetan sheep meat. The results suggest that Smac may regulate IAPs to promote mitochondrial apoptosis and muscle tenderization in Oula Tibetan sheep meat during postmortem aging. In summary, the evaluation of changes in Smac and IAPs concentrations may provide new insights into mitochondrial apoptosis and postmortem meat tenderization.

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CRediT authorship contribution statement

Jingyu Wang: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Ruina Zhao:** Supervision, Methodology, Investigation. **Yang Liu:** Supervision, Methodology, Investigation. **Tieying Hu:** Supervision, Investigation. **Xiaolong Li:** Methodology. **Long He:** Supervision, Methodology. **Zhaobin Guo:** Supervision. **Cheng Chen:** Supervision, Methodology. **Xixiong Shi:** Supervision, Conceptualization.

Declaration of competing interest

We confirm that no conflict interest conflict.

Data availability

The data presented in this paper are available upon request from the corresponding author.

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