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# Changes in calpain activity, protein degradation and microstructure of beef *M. semitendinosus* by the application of ultrasound

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ABSTRACT

In this investigation, samples were treated by ultrasonic probe (20 kHz) at an intensity of 25 W cm$^{-2}$ for 20 or 40 min, followed by aging at 4 °C for 1, 3 and 7 d. Ultrasound treatment significantly increased myofibrillar fragmentation index (MFI) and decreased Warner-Bratzler shear force (WBSF) at 3 and 7 d of postmortem aging ($P < .05$). Ultrasound-treated samples showed significantly greater intensities of autolyzed 76 kDa subunits and the lower intensity of intact 80 kDa form compared to control at 1 d of storage ($P < .05$). Significant difference was found between *semitendinosus* (ST) samples treated for both 20 and 40 min ($P < .05$). Ultrasound treated samples showed increased proteolysis during the postmortem storage as reflected by an increased degradation of desmin and troponin-T. These results showed that ultrasound treatment could improve the tenderness of beef ST muscle through regulating the calpain activation and protein degradation during postmortem aging.

## 1. Introduction

Tenderness is regarded as the most important palatability factor when consumers assess the quality of meat (Smith, Cannon, Novakofski, & Mckeith, 1991). Thus the changes in tenderness are closely related to the commercial value of the final meat product (Survey, 2000). Studies have indicated that the tenderization process occurring during the aging period is strongly attributed to the proteolysis of myofibrillar proteins, which is largely regulated by the endogenous protease including calpain along with cathepsin and caspase (Huff-Lonergan, Zhang, & Lonergan, 2010; Koohmaraie & Geesink, 2006).

Ultrasound is an innovative technology that has a wide range of applications in food industry. In meat processing, ultrasound can help in curing, marinating, cleaning and equipment sterilization. Ultrasound may induce compression and depression and produce micro-bubbles in the structure. The implosion of micro-bubbles causes cavitations which propagate shock waves of high energy throughout the tissue for a short period of time (Awad, Moharram, Shaltout, Asker, & Youssef, 2012; Got et al., 1999). Highly reactive radicals could be generated by the homolysis of liquid medium from transient cavitation (Ashokkumar, 2011). High-intensity ultrasound (20–100 kHz, 10–1000 W·cm$^{-2}$) can accelerate the mass transfer and chemical reactions through free radical production and micro-streaming generated by stable and transient cavitations (Kang, Gao, Ge, Zhou, & Zhang, 2017; Kang, Wang et al., 2016a; Mason, Paniwnyk, & Lorimer, 1996). Previous investigations have shown that lipid oxidation and protein oxidation may be initiated and accelerated by these free radicals and active oxygen molecules (Chang & Wong, 2012; Joerg, Francesco, Denisa, Desmondj, & Jamesg, 2009; Kang, Zou et al., 2016b). However, many researchers concluded that ultrasound could not show significant effects on the sensory properties of meat and meat products (Lyng, Allen, & Mckenna, 1998; Mcdonnell, Lyng, Arimi, & Allen, 2014).

Several studies have investigated the effects of power ultrasound on fresh meat quality. Lyng et al. (1998) found no significant effects when ultrasound treatment using a 20 kHz probe at the intensity of 62 W·cm$^{-2}$ was applied on beef *M. longissimus thoracis et lumborum* and ST for 15 s. Contrary to these findings, Jayasooriya, Torley, D'Arcy, and Bhandari (2007) reported an increased tenderness of beef *M. longissimus lumborum et thoracis* and ST muscles when they were exposed to high power ultrasound (24 kHz, 12 W·cm$^{-2}$, up to 240 s). Pohlman, Dikeman, Zayas, and Unruh (1997) applied ultrasound (20 kHz, 22 W·cm$^{-2}$) to beef pectoral muscles and found a reduced hardness with no significant effects of sonication time or storage.

However, there have been limited studies to be conducted to investigate the behind mechanism of ultrasound on beef tenderness during postmortem aging. In current study, the MFI, activation of calpain, post-mortem protein proteolysis, muscle microstructure and pH were measured to investigate the mechanism of ultrasound impacting

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on beef tenderness.

## 2. Materials and methods

### 2.1. Sample preparation and storage

Twelve 20-month-old yellow bull carcasses with live weight of 450 ± 15 kg were slaughtered humanely at a commercial meat processing company (Haoyue Halal Food Industry Co. Ltd., Kaifeng, China) under standard commercial conditions according to the National Standard of China. The ST muscles were excised from the left half of carcasses in a 4 °C chiller and then trimmed of subcutaneous fat and connective tissue. The beef muscles were selected within the pH range of 5.55–5.65, so as to ensure that the chosen samples were of normal expected quality. A digital pH-meter (HI 8424, Hanna, Italy) was used for the measurement of pH values.

### 2.2. Ultrasound treatment

At 24 h postmortem, all muscles were sliced into three blocks (80 mm × 70 mm × 25 mm) with the long axis parallel to fiber direction. The individual samples were vacuum-packaged in nylon and polyvinyl chloride bags and randomly divided into three groups. One group without ultrasound treatment served as control and the other two groups were sonicated at an ultrasound intensity of 25 $W \cdot cm^{-2}$ using an ultrasonic processor (VCX 750, SONICS, 20 kHz, USA) for either 20 or 40 min. The temperature was maintained at 5 ± 1 °C using a mixture of ice and water around a beaker. Following ultrasound treatment, the samples were stored for either 1, 3 or 7 d. The pH, WBSF, and transmission electron microscopy (TEM) were determined at each time point. The remaining beef samples were cut into small pieces and immediately frozen in liquid nitrogen and stored at −80 °C until required for further biochemical analysis.

### 2.3. pH analysis

The pH was measured at 1, 3 and 7 d during the process of aging using a HI 9125 portable digital pH meter (HANNA instruments, Cluj-Napoca, Romania). The pH meter was calibrated before measurements using standard buffers (pH 4.0 and 7.0) and adjusted to the actual temperature of sample measurement. Measurements were carried out in triplicate at random points of the samples.

### 2.4. Myofibrillar and sarcoplasmic protein extraction

Myofibrillar proteins were prepared using the method of Li et al. (2014) with some modifications. Samples were homogenized in PRB solution (2 mM $Na_4P_2O_7$, 1 mM $NaN_3$, 2 mM $MgCl_2$, 2 mM EGTA, 1 mM dithiothreitol (DTT), 100 mM KCl, and 10 mM Tris–maleate, pH 6.8) at a speed of 12,000 rpm for 10 s and three times with a 15 s interval cooling period between bursts. The homogenate was centrifuged at 1000*g* for 10 min at 4 °C (Avanti J-E, Beckman Coulter, USA). The pellet was washed three times with LSB buffer (composition was same as PRB but without pyrophosphate). After being suspended in EDTA (5 mM) and Tris-HCl (10 mM), the BCA Protein Assay Kit (Thermo, RD, USA) was used for analyzing the protein concentrations which were then adjusted to 8 mg protein/mL. Then, the myofibrils were mixed by vortexing with the treatment buffer (1:1, *v/v*) containing 20% glycerol (*v/v*), 125 mM Tris-HCl, 4% sodium dodecyl sulfate (*w/v*), 1% β-mercaptoethanol (*v/v*), and 0.01% bromophenol blue (*w/v*) (pH 6.8). The myofibrillar samples were heated at 95 °C for 5 min and then frozen at -80 °C until used for electrophoresis.

The isolation of sarcoplasmic protein was performed using the method described by Chen, Zhou, and Zhang (2015). Briefly, beef samples were homogenized in extraction buffer (pH 8.3) containing 100 mM Tris-base, 10 mM EDTA-$Na_2$, and 0.1% β-mercaptoethanol (MCE) with a polytron (IKA T25 digital ultraturrox Made in IKA, German) twice at 15,000 rpm for 30 s. Following centrifugation at 15,000*g* for 30 min at 4 °C, the concentration of supernatant was adjusted to 8 mg protein/mL. Then, the samples were mixed by vortexing with the treatment buffer (1:1, *v/v*) as used for myofibril extraction, heated at 95 °C for 5 min, and then kept in frozen storage.

### 2.5. Western Blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% (for μ-calpain and desmin) and 12.5% (for troponin-T) separating gels and 4% polyacrylamide stacking gels. The running buffer consisted of 0.1% SDS (*w/v*), 192 mM glycine, and 25 mM Tris. The changes in intensities of μ-calpain, desmin and troponin-T were determined on a Mini-PROTEAN Tetra cell (Bio-Rad Laboratories, Hercules, CA, USA) following the procedure of Fu et al. with some modifications (Fu et al., 2015). The gels were loaded with 45 μg proteins per well for calpain, 30 μg for desmin and 20 μg for troponin-T. A standard protein marker (6 μL) was used to determine the molecular weight of proteins. Samples before treatment at 0 d was as a standard reference for calpain and the untreated sample at 1 d was as a standard reference for desmin and troponin-T. The samples were run at a voltage of 90 V for 30 min following with a constant voltage of 120 V until the leading edge of the front was at the bottom of the gels. Then, proteins were immediately transferred to Bio Trace NT nitrocellulose transfer membranes (Pall, FL, USA) at 90 V for 90 min at 4 °C. The electro-blotted membranes were then blocked at room temperature in blocking buffer containing 20 mM Tris, 137 mM NaCl, 5 mM KCl, 5% skim fat dry milk powder (Solarbio, Beijing, China) and 0.1% Tween-20 (TBST) for 2 h. Membranes were incubated overnight at 4 °C in a 1:10,000 diluted monoclonal anti-μ-calpain primary antibody (MA 3-940; Affinity Bioreagents, Golden, CO, USA), 1:500 diluted monoclonal anti-desmin primary antibody (Abcam, Cambrigde, UK) and 1:1000 diluted monoclonal anti-troponin T primary antibody (T 6277, Sigma-Aldrich, Chemie GmbH, Germany) in TBST with gentle shaking. Excessive antibody was removed by washing the membrane in TBST for 25 min (5 min per wash) and then incubated for 2 h at room temperature with goat anti-mouse IgG HRP-conjugated secondary antibody (Bioworld Technology, Minnesota, USA) at a dilution of 1:5000 in TBST. After five washes with TBST, the membranes were treated with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, NY, USA). The band intensities were detected using an ImageQuant LAS 4000 imaging analyzer (GE Co., Ltd., Fairfield, CT, USA) and were quantified using Quantity One software.

### 2.6. MFI

MFI was determined following the procedure of Culler et al. at 1, 3 and 7 d postmortem (Culler, Prakash, Smith, & Cross, 1978) with some modifications. 1 g of minced muscle sample were homogenized twice (2 × 30 s) at 15,000 rpm in 20 ml ice-cold MFI buffer consisting of 20 mM $K_2HPO_4/KH_2PO_4$, pH 7.0, 100 mM KCl, 1 mM EDTA, 1 mM $NaN_3$, centrifuged at 1000*g* for 15 min at 4 °C. Remove the supernatant, the sediment was re-suspended and centrifuged for two times. Then the pellet was re-suspended in 10 ml of MFI buffer. The protein concentration of final suspension was adjusted to 0.5 mg/ml using the biuret method. The absorbance of the suspension was measured at 540 nm with a SpectraMax M3 spectrophotometer (Molecular Devices, CA, USA). For each sample, the mean value of duplicate absorbance readings was multiplied by 200 to give the MFI value.

### 2.7. TEM analysis

The microstructural changes occurring of raw and sonicated beef samples were evaluated by TEM after 1, 3 or 7 d of ultrasound treatment. Blocks of 1 mm × 1 mm × 2 mm were excised at the depth of