Animal (2012), **6:6**, pp 1018–1022 © The Animal Consortium 2011
doi:10.1017/S1751731111002047

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The effect of calcium chloride injection on shear force and caspase activities in bovine longissimus muscles during *postmortem* conditioning

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(Received 5 January 2011; Accepted 19 September 2011; First published online 23 November 2011)

Tenderness is considered as the most important quality determinant of meat. Calcium chloride application has been shown to improve tenderness by regulating endogenous proteinases. This study was designed to determine the effect of 300 mM calcium chloride injection on myofibrillar structures, caspase activities and shear force in longissimus muscles of bulls during postmortem *storage of 7 days. Myofibrillar fragmentation index was determined as an index of proteolysis occurring in muscle fibers and associated proteins. Maximum tenderness was observed at days 4 and 7 in both treated and control samples. The injection of calcium chloride significantly increased myofibrillar proteolysis and improved tenderness at* postmortem *days 4 and 7. The treatment reduced caspase-9 activity at 4 h and day 4, whereas those of caspase-8 and -3 activities at days 1 and 4 with respect to control. The improved tenderness and increased myofibril fragmentation with decreased caspase activities suggested that the proteolytic systems activated with calcium chloride injection possibly behave independent of the caspase system.*

Keywords: calcium chloride, caspases, conditioning, proteolysis, tenderness

Implications

Calcium chloride application in meat factory was thought to improve tenderness by increasing endogenous calpain activities. This manuscript was an original research to test whether calcium injection can accelerate tenderization by the caspase system triggering pathway. It provided a new thinking way about the meat conditioning process. Authors think that the studies of changes of caspase activities resulting from calcium injection would very likely trigger intense discussions about apoptosis, which was considered to be a new research model in the conversion of muscle into meat.

Introduction

Tenderness of beef gradually improves during *postmortem* storage at refrigerated temperatures (Lawrence *et al*., 2003). It is the major determinant of meat quality and it can be improved by various physical, chemical and biochemical means. Calcium chloride is added to muscle foods because of its ease of application, safety of use (approved by Food and Drug Administration) and as an additional source of calcium (Jaturasitha *et al*., 2004). It has been suggested to accelerate the tenderization process in the muscles (Morgan *et al*., 1991; Boleman *et al*., 1995; Kerth *et al*., 1995). Perez *et al*. (1998) reported improved tenderness of meat obtained from chicken, horse, cattle and rabbit by calcium chloride injection. The injection of calcium chloride has been thought to improve tenderness by regulating activities of the endogenous proteolytic system (Nurmahmudi and Sams, 1997; Polidori *et al*., 2000). The increase in myofibrillar fragmentation is an indicator of the amount of proteolysis and subsequent tenderization (Huff-Lonergan and Lonergan, 2008). Several enzyme systems have been reported to be involved in tenderization of meat; especially the calpain system has been extensively studied as a tool to accelerate *postmortem* tenderization by calcium ions (Gerelt *et al*., 2005). However, the effect of calcium ion injection on certain other proteolytic systems, like caspases, is yet to be explored.

Calcium ion has been indicated to activate caspases by two pathways. First, caspase-9 activity has been indicated to be sensitive to exogenous calcium ion (Menze and Hand, 2007).

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Second, calcium ion regulates caspases by inducing apoptosis (Tantral *et al.*, 2004; Chen *et al.*, 2005). In contrast, some studies indicated that caspases could be inhibited by the cleavage of calcium-dependent protease calpains (Wolf *et al.*, 1999; Chua *et al.*, 2000). Furthermore, the application of calcium ion has been reported to accelerate the depletion of ATP in *postmortem* muscle, whereas the availability of intracellular ATP is a prerequisite for the activation of caspases (Eguchi *et al.*, 1997; Delivoria-Papadopoulos *et al.*, 2007).

The caspase system has been shown to be involved in *postmortem* proteolysis and meat tenderization (Herrera-Mendez *et al.*, 2006; Cao *et al.*, 2010). However, the effect of added calcium chloride on caspase activities in muscle during *postmortem* conditioning needs further research. In this study, calcium chloride was injected into longissimus (LM) muscle taken from crossed bulls and was expected to accelerate the myofibrillar degradation by increasing caspase-regulated proteolytic activities, resulting in decreased shear force values depicting increased tenderization of beef muscle during *postmortem* conditioning.

Material and methods

Experimental design and sample processing

A total of 18 crossbred bulls (Simmental × local Chinese yellow cattle in Anhui province) of 22 to 24 months old having live weights of 450 ± 50 kg were procured from a local processing plant. Animals under study were handled humanely and slaughtered according to the requirements of National Standards of P.R. China 'Operating Procedures of Cattle Slaughter'. Animals were randomly divided into two equal groups. One group was treated with calcium chloride, whereas the other was taken as control. After 30 min of animal exsanguinations, LM muscles on the right side of the carcasses were taken from 5th to 11th thoracic vertebrae, exposed to air and stored at 4°C. LM muscles in the treatment group were then injected with a pre-chilled 300 mM calcium chloride solution at 10% (v/w) volumes of the meat weight at *postmortem* second hour, whereas the control group was not injected. Sampling was done by taking 10 g meat samples from each muscle, minced, wrapped in aluminum foil, frozen and stored in liquid nitrogen. These were used for determination of caspase activities and Myofibrillar Fragmentation Index (MFI) analysis at 4 h and at days 1, 2, 4 and 7 during storage.

Shear force measurement

Warner–Bratzler Shear force (WBSF) measurement was done in raw meat as described by Torrescano *et al.* (2003) after slight modification. Sample strips of (100 mm × 10 mm × 10 mm) from muscles stored at 4°C were taken. The fiber direction of the strips was parallel to the longitudinal axis. The peak force values were determined by placing the strips perpendicular to the longitudinal axis of the muscle fibers under Warner–Bratzler shear blade on XL1155 equipment (Xielikeji Co. Ltd, Herbin, China). The average peak force values of nine strips from each sample were considered as shear force values.

MFI values

MFI values were determined in liquid nitrogen frozen samples as described by Hopkins *et al.* (2004). Five hundred milligrams of the frozen muscles taken from liquid nitrogen were homogenized in 30 ml pre-chilled buffer. After homogenization, the myofibril suspensions were filtered, washed with 10 ml cold buffer and filtered again. The pellets of myofibrils were re-suspended into 10 ml buffer and centrifuged. This process was repeated thrice. The pellets were finally re-suspended in 10 ml cold buffer. The absorbance values of the diluted protein suspensions were measured immediately at 540 nm with a UV-2450 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The mean of the triplicate absorbance readings was multiplied by 150 to give index values for MFI values.

Measurement of the activities of caspase-9, -8 and -3

Caspase activities were determined as described by Du *et al.* (2004). Two hundred milligrams of frozen samples were taken from liquid nitrogen, pulverized and homogenized on ice in 0.5 ml lysis buffer comprising 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; pH 7.5), 10% sucrose, 0.1% nonyl phenoxypolyethoxylethanol-40, 10 mM dithiothreitol and a commercial protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA). Homogenates were subjected to three cycles of freeze–thaw (freezing was done at −20°C, while thawing at 4°C) before centrifugation at 18 000×**g** at 4°C for 30 min. Twenty microlitres of supernatant were combined with 0.2 ml of protease assay buffer (10% sucrose, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 100 mM HEPES pH 7.5); and then added 5 μl each reconstituted N-acetyl-Leu-Glu-His-Asp-7-amino-4-methylcoumarin (Ac-LEHD-AMC; catalog no. P444-0005, Biomol, Pennsylvania, USA), N-acetyl-Ile-Glu-Thr-Asp-7-amino-4-methylcoumarin (Ac-IETD-AMC; catalog no. P432-0005, Biomol, Pennsylvania, USA) and N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC; component no. 51-66081U, PharMingen, San Diego California, USA) substrates for caspase-9, -8 and -3, respectively. The mixture was incubated at 37°C for 1 h. Fluorescence values were determined by using excitation and emission wavelengths of 360 and 460 nm by a 96-Well Plate Reader (Tecan M200, Männedorf, Switzerland), respectively. Results were calculated as relative intensity of fluorescence per minute per milligram of meat.

Statistical analyses

Statistical analysis was performed by using the General ANOVA procedure of SAS 8.0 to determine the changes in values of WBSF, MFI and caspase-9, -8 and -3 activities within *postmortem* conditioning in both control and treated samples, whereas two samples *t*-test for means procedure was used to observe the significance of difference between

treatment and control groups. The level of significance used to justify a claim of a statistically significant effect was 0.05.

Results

WBSF

There was a significant decrease of WBSF values in both control and calcium chloride-injected samples ($P < 0.001$ and $P < 0.001$, respectively) from 4 h to day 1 and from days 2 to 4 (Figure 1). Maximum tenderness in both the treated and control samples was observed at days 4 and 7. Significant differences were observed between treated and control samples at days 4 and 7 ($P < 0.01$ and $P < 0.001$, respectively), which depicted that the injection of calcium chloride at 2 h improved the shear force values at days 4 and 7.

MFI values

The MFI values slightly decreased from 4 h to day 2, then increased significantly ($P < 0.001$) on days 4 and 7 of *post-mortem* conditioning (Figure 2) in both the control and treated samples. The calcium chloride treatment yielded significantly higher levels of myofibrillar fragmentation at days 4 and 7 as compared with those of control ($P < 0.05$ and $P < 0.01$, respectively).

Caspases

Caspase-9. There was a significant decrease in caspase-9 activity from 4 h to day 2 in both control and treated samples ($P < 0.001$; Figure 3), similar to those reported in *postmortem* porcine LM muscle by Kemp *et al.* (2006). The caspase-9 activity varied significantly among the treated and control samples at 4 h and day 4 ($P < 0.01$).

Caspase-8. Figure 4 shows that a significant decrease in caspase-8 activity occurred from days 1 to 2 in the control samples ($P < 0.001$), whereas there was an obvious decline of caspase-8 activities observed from the 4 h to day 2 in the treated samples ($P < 0.001$; Figure 4). The calcium chloride treatment decreased caspase-8 activities on *postmortem* days 1 and 4 ($P < 0.05$ and $P < 0.01$, respectively).

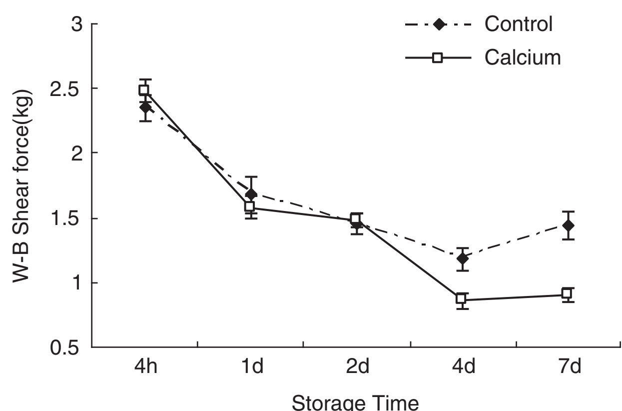

Figure 1 The effect of 300 mM calcium injection at the second hour *post mortem* on Warner–Bratzler Shear force values during conditioning for 7 days in longissimus muscles of bulls. The bar was defined as s.e.

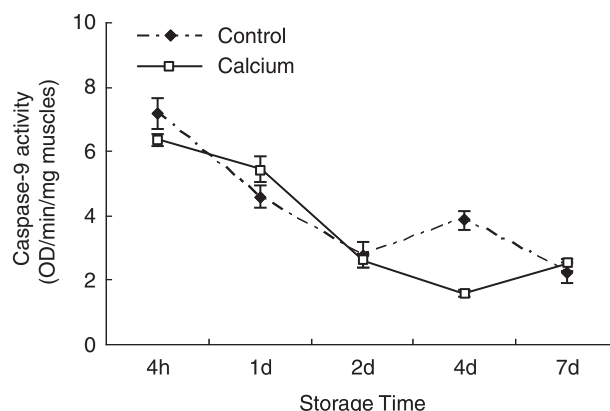

Figure 3 The effect of 300 mM calcium injection at the second hour *post mortem* on caspase-9 activities during conditioning for 7 days in longissimus muscles of bulls. The bar was defined as s.e.

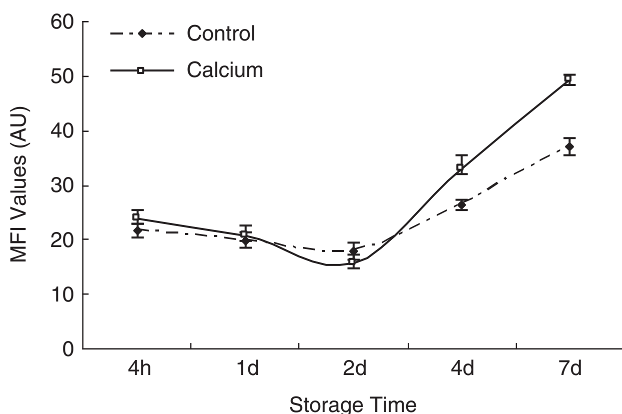

Figure 2 The effect of 300 mM calcium injection at the second hour *post mortem* on Myofibrillar Fragmentation Index values during conditioning for 7 days in longissimus muscles of bulls. The bar was defined as s.e.

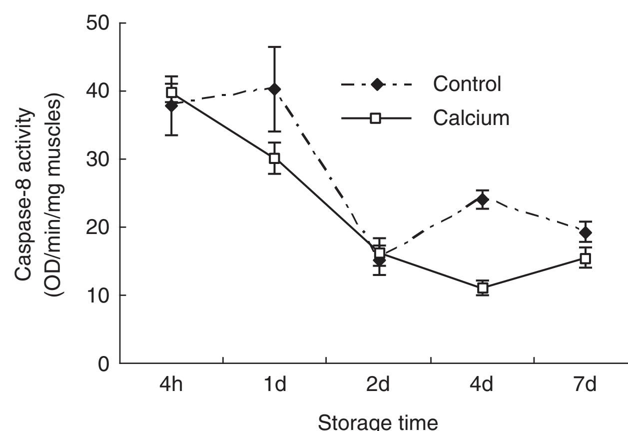

Figure 4 The effect of 300 mM calcium injection at the second hour *post mortem* on caspase-8 activities during conditioning for 7 days in longissimus muscles of bulls. The bar was defined as s.e.

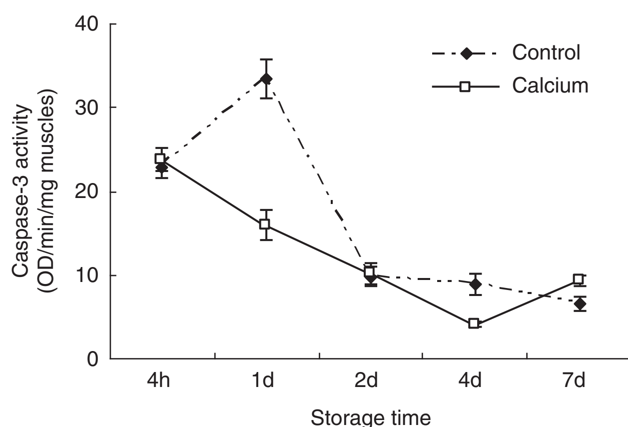

Figure 5 The effect of 300 mM calcium injection at the second hour *post mortem* on caspase-3 activities during conditioning for 7 days in longissimus muscles of bulls. The bar was defined as s.e.

Caspase-3. The caspase-3 activity significantly increased from 4 h to day 1, whereas subsequently decreased from days 1 to 2 in control ($P < 0.001$; Figure 5). Similarly, Kemp *et al*. (2006) reported that there was a general decrease in caspase-3/7 activity over storage with the highest activity at 2 h in pork. In addition, Underwood *et al*. (2008) indicated that caspase-3 activity decreased at days 3 and 10 *post mortem* when compared with 0 day in beef. Like caspase-8 activity, the caspase-3 activity in the treated samples significantly decreased from 4 h to day 4 ($P < 0.001$), with a significant decrease at days 1 and 4 ($P < 0.001$ and $P < 0.01$, respectively) with respect to control samples (Figure 5).

Discussion

WBSF

The reduction in WBSF values at day 1 showed that LM muscle after 1 day storage went through rigor phase and its toughest period. The data is consistent with the findings of Gonzalez *et al*. (2001) and Rees *et al*. (2002). The injection of calcium chloride at rigor decreased the shear force values at days 4 and 7, depicting the effectiveness of conditioning and calcium ion injection together as compared with conditioning alone in improving tenderness of LM. The results are in accordance with Morgan *et al*. (1991), Lawrence *et al*. (2003) and Jaturasitha *et al*. (2004) who injected 300 mM calcium chloride into pre-rigor bovine muscle resulting in significant improvement of meat tenderness.

MFI

The increase of MFI values on days 4 and 7 indicated arising myofibrillar proteolysis during the conditioning period. Our results showed that calcium chloride treatment produced higher levels of myofibril fragmentation during 4 and 7 days conditioning period than the levels achieved by the conditioning process alone. It means that the combination of conditioning and calcium ion injection is more effective in increasing action of endogenous proteolytic enzymes in *m. longissimus dorsi* than conditioning alone. Beekman *et al*. (1994) had similar findings for treated and control samples aged for 3 and 9 days in beef. The increase of myofibrillar proteolysis on days 4 and 7 resulting from calcium ion was responsible for the lower shear force values in treated samples, as many researchers have shown the correlation between MFI values and tenderness (Culler *et al*., 1978; Parrish *et al*., 1979).

Caspases

The decrease of caspase-9 activity in porcine LM muscle and that of caspase-3 in porcine LM muscle and bovine *m. longissimus thoracis* muscle were reported during *post-mortem* conditioning (Kemp *et al*., 2006; Underwood *et al*., 2008). During *postmortem* ischemia, intracellular ATP is rapidly degraded as a result of insufficient oxygen supply and rapid consumption of glycogen (Shen *et al*., 2006); whereas the availability of intracellular ATP is necessary for the activation of caspases. Liu *et al*. (2000) indicated that the depletion of ATP prevented the activation of caspase-9 and partially inhibited its ability to activate the terminal caspases and induce apoptosis. Delivoria-Papadopoulos *et al*. (2007) demonstrated that active caspase-9 density increased by 30%, 45% and 60% in the presence of ATP, cytochrome-*c* and ATP + cytochrome-*c*, respectively, in the hypoxic condition. Several reports have shown that ATP-dependent steps exist both upstream and downstream of caspase-3 activation in apoptotic signal transduction (Eguchi *et al*., 1997). Ferrari *et al*. (1998) reported that ATP was required for caspase-8 and -3 activation during anticancer drug-induced apoptosis. It is therefore plausible to suggest that the *postmortem* degradation in ATP was associated with the decrease of caspase activities. In addition, the application of calcium ion has been suggested to result in more rapid ATP depletion in *postmortem* chicken pectoralis major muscle (Young and Lyon, 1997) and porcine semimembranosus muscle (Miller *et al*., 1991). In this study, the reduced caspase-9 activity at 4 h and day 4, and those of caspase-8 and -3 at days 1 and 4 with respect to control were attributed to accelerated ATP depletion resulted from calcium treatment.

Lower caspase activities resulted from calcium chloride are also expected to occur through the cleavage of *post-mortem* activated calpains. The autolytic activation of the calpains resulting from calcium chloride injection has been shown in meat science (Pringle *et al*., 1999). Wolf *et al*. (1999) reported that purified porcine μ-calpain cleaved recombinant procaspase-9 and procaspase-3 to produce novel fragments, but without enzymatic activities. Chua *et al*. (2000) indicated that m-calpain cleaved procaspase-9 and decreased its activity. They suggested that calpains may act as negative regulators of caspase processing and apoptosis by effectively inactivating upstream caspases. It was concluded that improved shear force and enhanced myofibrillar proteolysis resulted from calcium ion in bovine muscle could not be associated with caspase activities.

Conclusion

The injection of 300 mM calcium chloride significantly improved the maximum tenderness, reduced caspase activities and increased myofibrillar proteolysis during *postmortem* conditioning. Therefore, the caspase system is most likely not involved in *postmortem* tenderizing activated by calcium injection.

Acknowledgement

We are greatful to W. Huang for her input and help in the analysis of the caspases activities data.

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