Meat Science 87 (2011) 165–174

Contents lists available at ScienceDirect

Meat Science

journal homepage: www.elsevier.com/locate/meatsci

Effects of camptothecin, etoposide and Ca^{2+} on caspase-3 activity and myofibrillar disruption of chicken during postmortem ageing

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ARTICLE INFO

Article history:
Received 17 June 2010
Received in revised form 26 September 2010
Accepted 4 October 2010

Keywords:
Caspase-3
Ageing
Ca^{2+}
Camptothecin
Etoposide
Apoptosis

ABSTRACT

Recently, a novel consideration has focused on the potential relationship of apoptosis and the protease caspases and the underlying mechanism for meat postmortem tenderization. In this study, apoptosis inducers, camptothecin and etoposide as well as Ca^{2+} were used to treat chicken muscle immediately after slaughter and follow the changes in caspase-3 activities and changes in the myofibrillar structures during 7 days of ageing. All three treatments resulted in significantly higher caspase-3 activities during storage ($p<0.05$), with the natural substrates, whereas Western blotting analysis of the α-spectrin cleavage product, 120 kDa peptide (SBDP 120), showed that Ca^{2+} was more effective than either camptothecin or etopside, and all were most active up to day 3 ($p<0.01$). According to SDS-PAGE, each treatment enhanced the accumulation of the 30 kD Troponin-T degradation product, especially during the first 3 days ($p<0.05$), and this was supported by the degradation of myofibrils observed by electron microscopy (TEM). TEM images showed the treatments resulted in enlargement of the I-bands and shrinkage of A-bands; however Z-lines were only slightly affected, even at day 7. The findings revealed that the three apoptosis inducers could increase myofibrillar dissociation and proteolysis during the first 3 days of chicken meat ageing. Because of the high activity of caspase-3 during the early postmortem period, it is possible that caspase-3 contributes to the conversion of muscle into meat.

1. Introduction

Tenderness, is regarded as the most important criterion for meat quality by most consumers (Brooks et al., 2000; Miller et al., 1995; Shackelford et al., 2001). Therefore a greater understanding of factors affecting meat tenderness would help meat producers provide consistently tender meat. Although the biochemical reactions which occur during postmortem tenderization remain controversial, it is believed that proteolysis of key myofibrillar proteins plays a major role in postmortem tenderization (Dransfield, 1994; Koohmaraie, 1992, 1994). Tenderization during ageing is strongly attributed to the proteolytic breakdown of key myofibrillar proteins, titin, nebulin, filamin, desmin and troponin-T (Huff-Lonergan et al., 1996; Koohmaraie, 1992); the most pronounced degradation is that of troponin-T (Bowker, Fahrenholz, Paroczay, Eastridge, & Solomon, 2008; Penny & Dransfield, 1979). The presence of a 30 kDa component, a fragment arising from troponin-T (Ho, Stromer, & Robson, 1994; Huff-Lonergan et al., 1996; Penny & Dransfield, 1979), has been reported in aged beef (Negishi, Yamamoto, & Kuwata, 1996), chicken (Samejima & Wolfe, 1976), pork (Koohmaraie, Whipple, Kretchmar, Crouse, & Mersmann, 1991) and lamb (Whipple & Koohmaraie, 1992). Furthermore, the most noticeable change occurring in myofibrillar proteins during the ageing process is the degradation of troponin-T and the concomitant appearance of a 30 kDa polypeptide which is a useful indicator of postmortem tenderization (Huff-Lonergan et al., 1996; Hughes, Geary, Dransfield, McSweeney, & O'Neill, 2001; Miros et al., 2008; Muroya, Oe, Nakajima, Shibata, & Chikuni, 2009; Muroya et al., 2004; Negishi et al., 1996; Olsen, Parrish, Dayton, & Goll, 1977; Penny & Dransfield, 1979; Tsitsilonis et al., 2002; Voelter et al., 2000). As a general consideration, meat tenderizing is a result of weakening of myofibrillar structures and associated proteins due to endogenous proteolytic enzymes (Sentandreu, Coulis, & Ouali, 2002). Considerable research has revealed that during the postmortem period, proteolysis is mainly due to calpains and cathepsins, with the calpain system being considered the major contributor to meat tenderization during the early stages (Koohmaraie, 1992, 1996; Koohmaraie & Geesink, 2006; Olsen et al., 1977; Penny, Voyle, & Dransfield, 1974). Nevertheless, it is thought that these systems are not the sole proteolytic determinants of meat quality and that meat tenderization is a multienzyme system in which other proteases such as proteasome and caspases may

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0309-1740/$ – see front matter.
doi:10.1016/j.meatsci.2010.10.002

contribute (Herrera-Mendez, Becila, Boudjellal, & Ouali, 2006; Ouali et al., 2006).

Following slaughter, blood is lost from the tissues and any remaining oxygen is consumed by the cells within a short time. The muscle fibres will inevitably become hypoxic–ischemic leading to an apoptotic–necrotic state (Nakajima et al., 2000). One line of thought is that following exsanguination, muscle cells are committed to cell death during the ageing process; hence apoptosis should be considered before rigor mortis (Herrera-Mendez et al., 2006; Ouali et al., 2006). Apoptosis is a normal physiological phenomenon that occurs in proliferating and constantly renewing tissues as part of the turnover process. Caspases, a family of cysteine proteases are pivotal for apoptosis, and are activated early in pathological events associated with hypoxic–ischemic, which is similar to the conditions that occur postmortem (Gustafsson & Gottlieb, 2003). Kemp, Bardsley, and Parr (2006) investigated the expression of caspases in different skeletal muscle types and proposed that the protease family of caspases was associated with meat tenderization. The importance of this system as a contributor to postmortem tenderization has been debated (Underwood, Means, & Du, 2008). As there are significant similarities between meat ageing and apoptosis, the role of caspases in meat tenderization should be considered. Caspase-3, the most vital member of the caspases, is a member of the interleukin-1β-converting enzyme family of cysteine proteases which trigger the execution phase of apoptosis (Chowdhury, Tharakan, & Bhat, 2008; Yuan, Shaham, Ledoux, Ellis, & Horvitz, 1993). Although, much research has focused on the effect of caspase-3 on proteolysis of myofibrils during postmortem conditioning, it has not been established that caspase-3 is an essential contributor to meat tenderization. Kemp et al. (2006) demonstrated that caspase-3 activity increased during the early stages postmortem, but then decreased with time and was negatively related to Warner–Bratzler shear force. A recent study provided further evidence of the potential role of caspases in meat tenderization by degradation of myofibrillar proteins using recombinant caspase-3 (Kemp & Parr, 2008). Also, Huang, Huang, Xu, and Zhou (2009) used a caspase-3 selective inhibitor, DEVD-CHO (N-acetyl-Asp-Glu-Val-Asp-CHO) and showed that it significantly inhibited degradation of the skeletal proteins titin, nebulin, desmin and troponin-T, whereas the activity of the calpains was not influenced. Therefore, it is proposed that the degradation of muscle proteins should not be exclusively attributed to the calpain system and that caspase-3 may be a protease involved in postmortem tenderization.

Camptothecin is an alkaloid isolated from *Camptotheca acuminata* (family *Nyssaceae*) which possesses strong anti-tumor activities against a wide range of tumors (Lin, Wilkinson, & Farber, 1998). Camptothecin is associated with the ability to form intracellular DNA–protein adducts and can reversibly inhibit nuclear topoisomerase I by binding specifically to, and stabilizing the topoisomerase–DNA covalent complex (Grądzka, Skierski, & Szumiel, 1998; Murren, Beidler, & Cheng, 1996). Etoposide, a derivative of podophyllotoxin, well known as an anticancer agent, is an inhibitor of topoisomerase II and a potent inducer of breakage of DNA strands by forming a cleavable complex between topoisomerase II and DNA (Floros, Thomadaki, Florou, Talieri, & Scorilas, 2006; Lee, Enomoto, Koshiba, & Hirano, 2009; Lee et al., 2007; Sjöblom, West, & Lähdetie, 1998). It has long been considered responsible for DNA degradation which is frequently observed during apoptosis, and these two agents have been shown to induce apoptotic cell death in various normal and cancer cells. As Ca^{2+} is a key cellular regulator and crucial second messenger in cell signal transduction, it is not surprising that Ca^{2+} has been strongly implicated in induction and regulation of the apoptosis signaling pathways. Moreover, there is considerable evidence that the kinetics of Ca^{2+} release is correlated with the onset of apoptosis, which suggests that Ca^{2+} may play a direct role in apoptosis induction (Hajnóczky et al., 2006; Nicotera & Orrenius, 1998; Orrenius, Zhivotovsky, & Nicotera, 2003; Pörn-Ares, Ares, & Orrenius, 1998; Trump & Berezesky, 1996). In addition, increased levels of intracellular Ca^{2+} trigger activation of Ca^{2+}-dependent enzymes culminating in cell death, and this increase has been postulated to exercise a regulatory function in apoptosis (McConkey, Zhivotovsky, & Orrenius, 1996; Nicotera & Orrenius, 1998; Trump & Berezesky, 1996). A few reports highlight the role of caspases during meat tenderization; however, these lack focus on the apoptotic mechanism in association with execution target caspase-3 during postmortem ageing. There is still much debate regarding the role of caspase-3 in myofibrillar proteolysis. The present study was designed to determine the changes in caspase-3 activity during postmortem ageing of chicken muscle resulting from addition of apoptosis inducer chemicals camptothecin, etoposide and Ca^{2+} and then to investigate their role in proteolysis of myofibrillar structures.

2. Materials and methods

2.1. Chemicals

The following were purchased from Amresco Inc. (Solon, OH 44139 USA): phenylmethylsulfonyl fluoride (PMSF), sodium dodecyl sulphate (SDS), 2-amino-2(hydroxymethyl)-1,3-propandiol (Tris), Triton X-100, DL-dithiothreitol DTT), calcuim chloride ($CaCl_2$), dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid disodium salt, and dihydrate (EDTA Na_2).

Camptothecin and etoposide were obtained from BioVision Research Products (Mountain View, CA, USA), protease inhibitor cocktail tablet (Roche Diagnostics GmbH, Mannheim 68298 Germany), BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL 61105 USA), anti-Fodrin (spectrin) monoclonal antibody (Millipore, Temecula, CA 9290 USA), anti-mouse IgG, HRP-linked Antibody (Cell Signaling Technology, Inc. Danvers, MA 01923 USA), DAB (Sigma-Aldrich, St. Louis, USA), and the end-point Apo-One Homogeneous Caspase-3/7 Assay (Promega, Madison WI 53711-5399 USA).

All other chemicals and reagents were obtained from commercial sources at the highest purity available.

2.2. Animals and treatment

Four Yellow-feathered chickens, a Chinese native breed (female, 45 days, 2.0–2.5 kg), from the experimental station of Nanjing Agricultural University, were cared for and slaughtered as outlined in the guide for the care and use of experimental animals (Animal Experimental Special Committee of NAU). Chickens were slaughtered and breast muscles (*Musculus pectoralis superficialis*) were rapidly removed and cut into small pieces (about 0.2 g), and thoroughly mixed. A sample from each chicken was immediately (0 day) snap-frozen in liquid nitrogen (<10 min) and stored until required for subsequent analysis. The remainder of each of the individual batches of chopped muscle was subdivided into three fractions and allocated to treatments as follows: one portion received no treatment (control) and three samples were soaked in solutions containing either 40 mg/ml camptothecin, 40 mg/ml etoposide, or 0.05 M $CaCl_2$ in the ratio 1:2 (meat:solution; w/v), and then stored at 4 °C for 1, 3, 5 and 7 days. At the end of each storage period the samples were taken individually and stored in liquid nitrogen until required.

2.3. Transmission electron microscopy

Thin strips of muscle were excised from the breast and all specimens were cut into 1 mm × 1 mm × 2 mm sections and fixed with 3.0% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 initially at room temp, and then at 4 °C overnight. Muscle samples were then washed for several hours in sodium cacodylate buffer (0.1 M, pH 7.4), and then, fixed in 2% osmium tetroxide in the same buffer for 2 h after which they were rinsed briefly in 2% sodium acetate. A negative stain

with 2% uranyl acetate in water was applied for 1 h in a foil covered bottle in a fume hood. Samples were then dehydrated in ethanol using the following sequence: 50% for 10 min; 75% for 10 min; 95% for 10 min; and 100% (dry) for 10 min. Ultra-structural changes in myofibrils were observed and photographed using a transmission electron microscope H-7650 (Hitachi Ltd., Tokyo, Japan) operated at 80 kV.

2.4. Preparation of sarcoplasmic and myofibril protein

Extraction of sarcoplasmic proteins was performed according to Koumura et al. (2008) with modifications. The meat samples were homogenized in 2.0 ml/g tissue in ice-cold lysis buffer containing 25 mM Tris–HCl (pH 7.6), 150 mM NaCl, 50 mM EDTA, 1 mM DTT, 1 mM PMSF, 5 mM sodium fluoride (NaF), 1% Triton X-100 and protease inhibitor cocktail (10 ml/tablet) using a homogenizer (S10, Ningbo China) at 25,000 rpm for 6 periods of 10 s, with a 10 s cooling period between bursts. The homogenates were then centrifuged at 14,000 *g* for 40 min at 4 °C, and the supernatants collected and adjusted to equal protein concentrations following protein determinations using a BCA Protein Assay Kit. The purification of myofibrils from muscle tissues was performed at 4 °C as described by Huang et al. (2009) with minor revisions. The pure myofibrils were mixed with buffer (125 mM Tris–HCl, pH 6.8, 4% SDS) heated in a 50 °C water bath for 20 min, and then centrifuged for 45 min at 16,000 *g*, 25 °C. Protein concentration was determined with the BCA Protein Assay Kit.

2.5. Determination of caspases-3 activity

Caspase-3 activity was assessed using the end-point Apo-One Homogeneous Caspase-3/7 Assay (Promega, UK) according to the manufacturers' protocol. The caspase reagent was added to the sarcoplasmic proteins of the supernatant in a 1:1 ratio and incubated at 37 °C for 4 h. Reaction products were assessed using a Tecan fluorescence spectrometer (Tecan, Switzerland) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

2.6. SDS-PAGE and Western blot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 4% stacking gel and 12.5% separating gel in a discontinuous buffer system as described by Huang et al. (2009). An appropriate aliquot of each sample was diluted to 1.5 mg/ml using a buffer containing 0.5% β-mercapto-ethanol, 10% glycerol and 0.02% bromophenol blue. Samples were well mixed and heated at 50 °C for 20 min. Each lane contained a total of 20 μg protein. The analyses of spectrin and troponin-T degradation were determined on a Mini-PROTEAN Tetra cell (Bio-Rad Laboratories, Hercules, CA), and the gels (9 cm wide × 8 cm tall) were loaded with 40 μg per well of protein for α-spectrin and 20 μg for troponin-T. They were run at a constant voltage of 80 V for 45 min, and then a constant voltage of 120 V for nearly 2 h. Then, the troponin-T gels were fixed and stained for protein with 0.1% Coomassie brilliant blue R-250 in 45% (v/v) methanol and 10% (v/v) acetic acid and destained by diffusion of unbound dye from gels by extensive washing with 10% (v/v) methanol containing 10% (v/v) acetic acid. α-Spectrin degradation was analyzed by Western blot analysis, and gels were immediately transferred to PVDF membranes (Millipore, Temecula, CA 9290 USA) using a Semi-Dry Transfer Cell (Bio-Rad Laboratories, Hercules, CA) at a constant current of 2.5 mA/cm^2 for 50 min. The electro-blotted membrane was then blocked overnight at 4 °C in blocking buffer (TTBS: 0.1% Tween 20, 20 mM Tris, 137 mM NaCl, 5 mM KCl and 5% skim milk powder) for 90 min. Membranes were rinsed in TTBS and incubated overnight at 4 °C in a 1:500 diluted primary antibody in TTBS which containing 5% BSA and 0.1% Tween-20, with gentle shaking. Excess antibody was removed by washing the membrane in TTBS and it was subsequently incubated for 1 h at room temperature with horse anti-mouse IgG HRP-conjugated secondary antibody diluted 1:2500 in TTBS. After several washes in TTBS, bands were visualized with DAB.

The gels were photographed with a Gel Doc XR™ System (Bio-Rad Laboratories, Hercules, CA) and membranes were scanned (Scan-Maker 4100, MICROTEK, Shanghai China), and then the intensities of gels and Western blotting bands were quantified by Quantity One software (Bio-Rad Laboratories, Hercules, CA) within the calibration range.

2.7. Statistical analyses

Data are shown as mean ± SD. The data were analyzed using the SPSS statistical package programmer by one-way ANOVA, and differences among the individual means were compared by Duncan's multiple range tests, $p<0.05$ was the level for significance.

3. Results

3.1. Caspase-3 activity

Caspase-3 activity was measured by a standard fluorescence-based assay which cannot distinguish between caspases-3 and -7 activities. The activity at 0 day was the highest in all treatments. A sharp decline was observed within 24 h, however, no further significant variation was observed at 3, 5 and 7 days in any of the four groups and the decline rate remained more or less constant during 7 days ageing (Table 1). Compared to the control, the caspase-3 activities with camptothecin and etoposide were significantly higher ($p<0.01$), as was their activity in the presence of Ca^{2+} ($p<0.05$). During the 7 days postmortem, a decreasing trend was observed for the caspase-3 activity of the control, however for all other treatments, the activities were higher than the control (Table 1).

3.2. Determination of the relative activities of caspase-3 in the degradation of α-spectrin

Caspase-3 mediated cleavage of α-spectrin generates 150 and 120 kDa degradation products (SBDP150 and SBDP120, respectively), and α-spectrin can also be cleaved by calpains producing another 150 kDa peptide and a 145 kDa peptide (SBDP145). In this study Western blots probed with anti-α-spectrin detected immunopositive bands of intact 240 kDa, α-spectrin, and cleavage products, 150 and 120 kDa from α-spectrin. SBDP145 was not detected by this antibody (Fig. 1(A, B, C)). Treatment of muscle with camptothecin, etoposide or Ca^{2+} led to the degradation of α-spectrin resulting in a major cleavage product of 150 kDa, together with a minor 120 kDa fragment (Fig. 1(A, B, C)). The bands of intact 240 kDa α-spectrin and SBDP150 were clearly evident on 0 day, but during postmortem ageing, α-spectrin was further cleaved into SBDP150 (Fig. 1(A, B, C)). Although the

Table 1
Caspase-3 activities of control, camptothecin, etoposide and Ca^{2+} treated chicken meat using homogeneous Apo-ONE assay (Promega) when stored at 4 °C for 0, 1, 3, 5 or 7 day.

Caspase-3 activity	Control	Camptothecin	Etoposide	Ca^{2+}
0 day	261.92 ± 0.03	–	–	–
1 day	26.00 ± 3.10^a	93.97 ± 9.97^b	95.9 ± 8.41^b	37.16 ± 9.13^c
3 day	9.47 ± 0.13^a	53.67 ± 5.64^b	63.46 ± 3.98^b	25.33 ± 0.25^c
5 day	7.88 ± 0.17^a	50.17 ± 4.35^b	42.10 ± 6.39^b	22.07 ± 0.05^c
7 day	6.18 ± 0.45^a	56.47 ± 3.90^b	50.18 ± 7.73^b	23.45 ± 2.02^c

Note: Values are expressed as absorbance (arbitrary units) per mg/ml protein concentration. Each value represents the mean ± SD, n = 4.
^{a,b,c,} : Means within a row with different superscripts are significantly different ($p<0.05$).

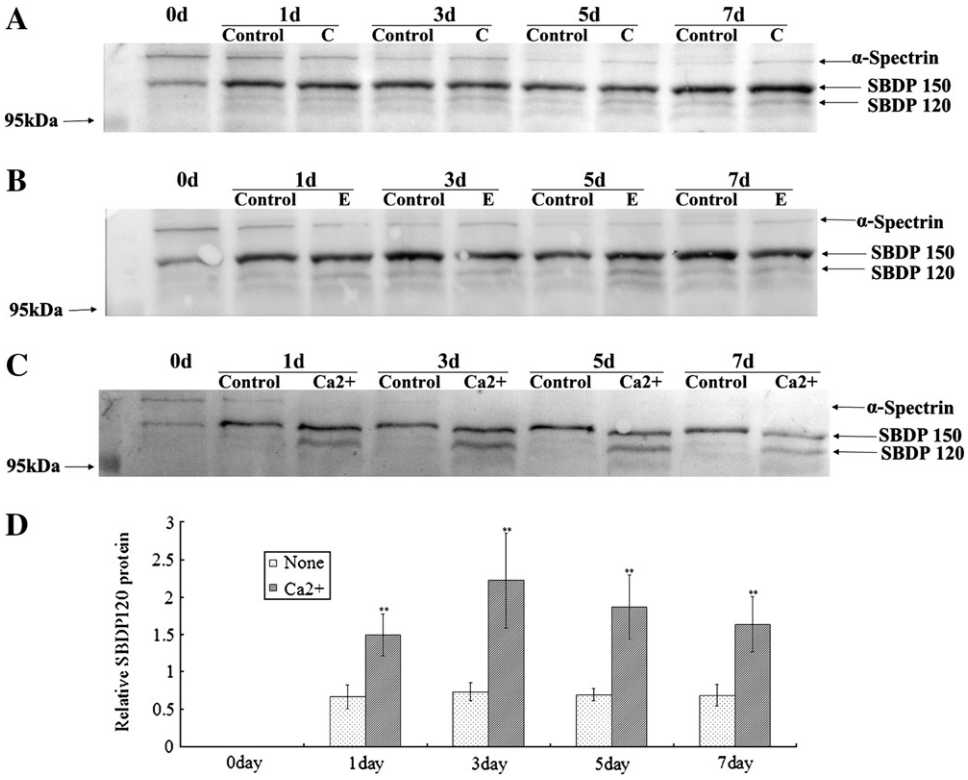

Fig. 1. Cleavage of α-spectrin by chicken breast muscle preparations in the presence (C) and absence (Control) of (A) 40 mg/ml camptothecin; presence (E) and absence (Control) of (B) 40 mg/ml etoposide; and presence (Ca2+) and absence (Control) of (C) 0.05 M $CaCl_2$ when stored at 4 °C for 0, 1, 3, 5 and 7 days as shown by Western blots with anti-α-spectrin; together with the relative values of 120 kDa cleavage products of α-spectrin (SBDP120) during 7 days of storage at 4 °C. Forty microgram of protein from each sample was loaded onto each lane (A, B, and C). Relative values were calculated as the blot intensity of the full-length α-spectrin (240 kDa) at 0 day (D). Mean ± SD (n = 4). **: $p<0.01$ vs. control.

SBDP120 fragments showed up as very light bands, on close examination it was evident that compared with the controls, camptothecin and etoposide treatments of the meat resulted in increased amounts of this cleavage product at each storage time, with the possible exception of etopside on day 7 (Fig. 1(A, B)). Contrarily, the intensity values of SBDP120 were very high with Ca^{2+} treatment (Fig. 1(C)), and there were statistically significant differences between the Ca^{2+} treatment and the control in the 1, 3, 5 and 7 day-samples when using intact 240 kDa as a marker in each Western blot image ($p<0.01$) (Fig. 1(D)). It showed similar low intensity values of SBDP120 in the control during 7 days ageing, while Ca^{2+} treatment increased the intensity of the SBDP120 immunopositive band which peaked at day 3, with levels at 5 and 7 days somewhat lower, but still higher than at day 1 (Fig. 1(D)).

3.3. Effects of camptothecin, etoposide and Ca^{2+} on the postmortem degradation of troponin-T

SDS-PAGE of the myofibrillar samples clearly showed the formation of the 30 kDa fragment from Troponin-T during ageing, and confirmed that treatment with camptothecin, etoposide or Ca^{2+} affected the accumulation of the 30 kDa Troponin-T fragments (Figs. 2, 3, and 4). This demonstrated that the abundance of the 30 kDa Troponin-T fragment increased with ageing and was higher in camptothecin, etoposide and Ca^{2+} treated samples during 1 and 3 days of ageing compared to the control (Figs. 2, 3, and 4). Both the camptothecin and etoposide treatments caused the 30 kDa Troponin-T fragment to be significantly higher at day 1 ($p<0.05$) (Figs. 2 and 3) and at day 3 ($p<0.01$) (Figs. 2 and 3). Ca^{2+} treatment also resulted in an increase at both 1 and 3 days ($p<0.01$) (Fig. 4). However, the amounts of 30 kDa troponin-T were slightly higher with Ca^{2+} treatment at days 5 and 7 and with camptothecin at day 5 but these differences were not significant. It can be concluded that all three treatments affected postmortem proteolysis of chicken meat during the first 3 days, but was ineffective after that time. Although some treatments decreased the amount of 30 kDa troponin-T fragment after 5 and 7 days, there was no significant difference in the extent of degradation.

3.4. Transmission electronmicrographs of the myofibrils

Electron micrographs of the longitudinal sections of treated and control chicken muscle samples are shown in Fig. 5. In controls, the A-bands and I-bands were clearly distinguishable, the Z-lines were intact and M-lines were visible throughout the whole ageing period. In addition, a number of ultra-structural changes occurred during the 7 day ageing period (Fig. 5(A, B, C)). The visual fragmentation of myofibrils appeared at varying rates depending on the particular treatment, but in particular, the I-bands showed the greatest changes under all treatments. Broadening of I-bands was observed in the treated samples from 1 day compared with the control which only occurred at day 7 (Fig. 5(C, D, G, J)). When the ultra-structure of muscle was examined after treatment with camptothecin and Ca^{2+}, rapid structural changes became apparent which led to the disintegration of the myofibrillar scaffold (Fig. 5(D–F and J–L)). Moreover, for those samples treated with camptothecin or Ca^{2+} there was significant disorganization of the sarcomeres as seen from the altered I-bands, the loss of actin filaments, and an overall loosening of alignment structure of the myofilaments at 1, 3, 5 and 7 days. Also, some gaps or breakdown in the Z-lines occurred (Fig. 5(D–F and J–L)). Thus compared with the control, camptothecin and Ca^{2+} resulted in significant changes in the structures of the myofibrils by breaking down the main structural elements. At the concentrations used, their effects were similar except that camptothecin exhibited a greater disruption of the A- and M-lines (Fig. 5(D–F and J–L)). The severely disordered A-bands and distorted I filaments were observed in the

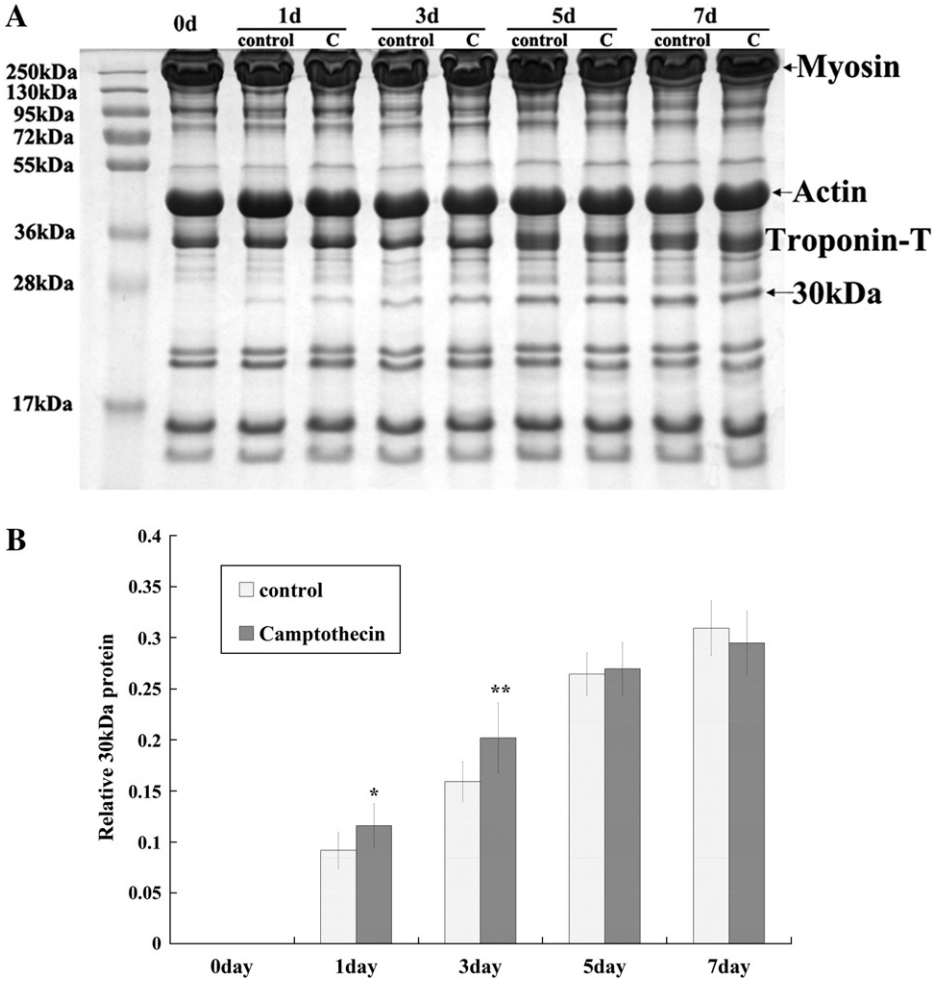

Fig. 2. Proteolysis of chicken breast muscle preparations in the presence (C) and absence (control) of 40 mg/ml Camptothecin when stored at 4 °C for 0, 1, 3, 5 & 7 days as shown by (A) SDS-PAGE of myofibrillar proteins (20 μg of protein per lane) together with the relative values (B) of the 30 kDa TnT fragment during 7 days of storage at 4 °C. Relative values were calculated as the blot density of the actin in the same lane of each gel across the 7 days. Mean ± SD (n = 4). **: $p<0.01$ vs. control.

camptothecin-treated samples from day 3 and by day 7, nearly all structures were lost (Fig. 5(E, F, L)). However, addition of etoposide only resulted in several modifications of the ultra-structure of the sarcomere, such as loosening of the I-bands, but overall, the myofibrils looked almost intact with the sarcomeres being unchanged even at day 7 (Fig. 5(G, H, I)). All in all, at day 3 all the treatments had affected myofibrillar ultra-structures, almost as dramatically as observed at day 7 (Fig. 5(D–F, G–H, I and J–L)). In this study it has been shown that each of the three chemical treatments resulted in a large effect on both the I- and A-bands but left the Z-lines mainly intact except for some minor ruptures at the connection sites of adjacent myofibrils (Fig. 5).

4. Discussion

Following slaughter, all muscle fibres will become hypoxic–ischemic, and the muscle tissue will develop a state of apoptosis. Intriguingly, most programmed cell death responses finally lead to caspase activation which might function as central integrators of the death pathway (Grunewald, Sharma, Paasch, Glander, & Agarwal, 2009; Pinton, Ferrari, Virgilio, Pozzan, & Rizzuto, 2001; Ventimiglia et al., 2001). In this investigation, Ca^{2+} and two types of topoisomerase inhibitors, camptothecin and etoposide acted as apoptosis inducers in order to examine their effect on the activity of caspase-3, and thus improve the tenderness of chicken meat during postmortem ageing. Camptothecin, as the major topoisomerase inhibitor, induces apoptosis in various cells, such as cardiomyocytes maintained in culture (Kong & Rabkin, 1999), myelogenous leukemia cells (Traganos, Seiter, Feldman, Halicka, & Darzynkiewicz, 1996), thymocytes (Bino, Bruno, Yi, & Darzynkiewicz, 1992), lymphocytes (Williams, 1991), and human myelogenous HL-60 cells (Bino et al., 1992). In addition, etoposide induces apoptosis of several malignant cell lines, such as the original human leukemia cell line, human T cells, mouse thymocytes, HL-60 and activated macrophages (Finucane, Waterhouse, Amarante-Mendes, Cotter, & Green, 1999; Joel, 1996; Nakama et al., 2001). It is also widely reported that an increase in the concentration of intracellular Ca^{2+} induces apoptosis in various cell models, and the critical characteristic of the apoptotic Ca^{2+} signal is a sustained increase of intracellular Ca^{2+} (Berridge, Bootman, & Lipp, 1998; Mathiasen et al., 2002; McConkey & Orrenius, 1997; Orrenius et al., 2003; Sergeev, 2004). There are numerous reports demonstrating the relationship between apoptosis and changes of intracellular Ca^{2+} concentration. Twiner, Chidiac, Dixon, and Trick (2005) showed that extracellular organic compounds from cultures of *H. akashiwo* acutely increased intracellular Ca^{2+} in cells that had been induced for apoptotic cell death. Misonou, Asahi, Yokoe, Miyoshi, and Taniguchi (2006) demonstrated that acrolein increased intracellular Ca^{2+} levels leading to apoptosis of human umbilical vein endothelial cells. It has also been shown that poly-methoxylated flavones induce Ca^{2+}-mediated apoptosis in breast cancer cells (Sergeev, Li, Colby, Ho, & Dushenkov, 2006). In the present study, unlike other work using cell lines in culture, muscle from freshly slaughtered chicken meat was used for comparing the effects of the drugs and high Ca^{2+}

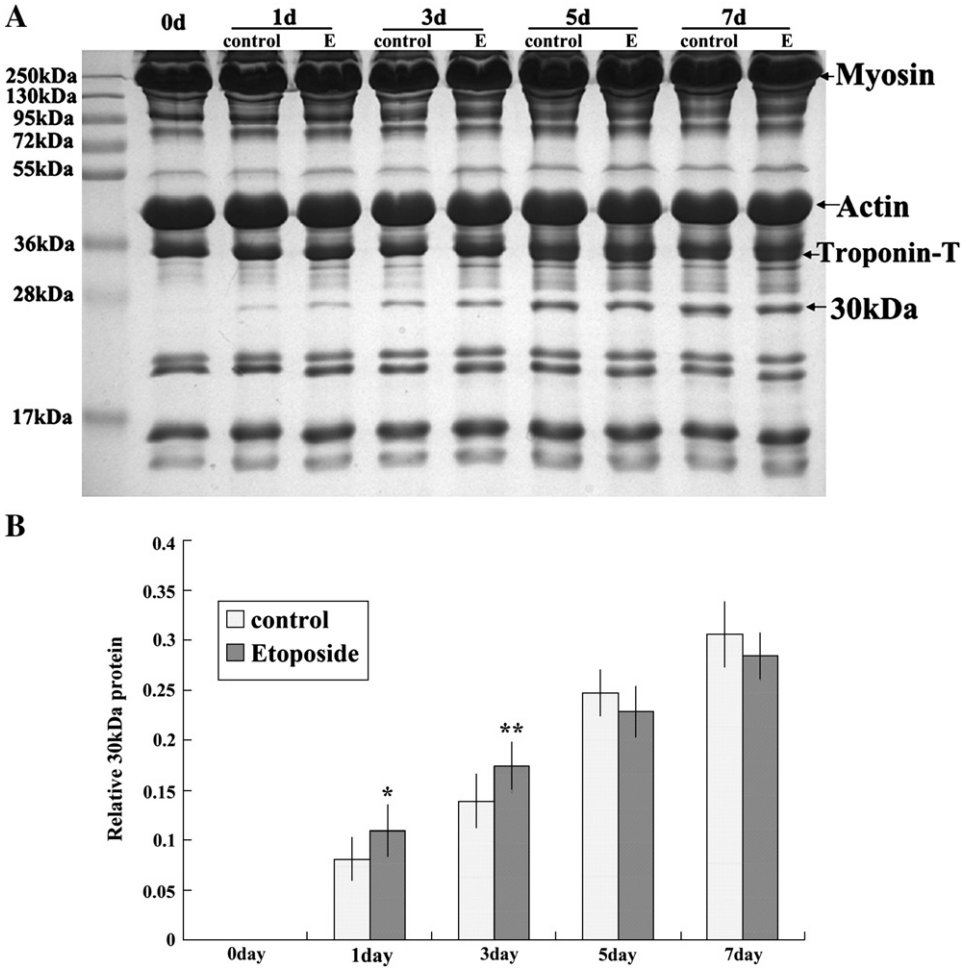

Fig. 3. Proteolysis of chicken breast muscle preparations in the presence (E) and absence (control) of 40 mg/ml Etoposide when stored at 4 °C for 0, 1, 3, 5 and 7 days as shown by (A) SDS-PAGE of myofibrillar proteins (20 μg of protein per lane) together with the relative values (B) of the 30 kDa TnT fragment during 7 days of storage at 4 °C. Relative values were calculated as the blot densitometry of the actin in the same lane of each gel across the 7 days. Mean ± SD (n = 4). **: $p<0.01$ vs. control.

concentrations on caspase activities. The results showed that each treatment significantly affected caspase-3 activity over the 7 days ageing period (Table 1).

This work showed that all the three treatments caused caspase-3 activity to increase significantly compared with the control (Table 1), but interestingly, only the addition of Ca^{2+} resulted in the generation of the 120 kDa degradation product (SBDP 120), which has been used as a marker for caspase-3 mediated proteolysis and apoptosis. On the other hand, the apoptosis chemicals camptothecin and etoposide led to extremely higher caspase-3 activities (Table 1), but failed to produce the 120 kDa fragment expected from the caspase-3 cleavage of α-spectrin (Fig. 1(A, B)). The degradation of α-spectrin during apoptosis could significantly compromise membrane permeability and cytoskeletal integrity, leading to structural changes that are associated with meat tenderization (Taylor, Gessink, Thompson, Koohmaraie, & Goll, 1995; Wang, 2000). As indicated previously, α-spectrin is broken down by both caspases and calpains during cell death (Sic & Mark, 1999); however the cleavage products differ, depending on the particular enzyme involved. The actual breakdown pattern of α-spectrin into the spectrin-breakdown products (SBDP) is regarded as indicative for either caspase or calpain activation in cell death. Intact α-spectrin migrates as a 240 kDa peptide on Western blots, and the 150 kDa fragment is indicative of caspase and calpain activities. However, the 145 kDa fragment results from calpain activity whereas the 120 kDa fragment is believed to result from caspase activity (Nath et al., 1996; Pike et al., 1998; Sidhu, Tuor, & Del Bigio, 1997). In fact, caspase-3 is specifically responsible for the production of SBDP120 (Jänicke, Ng, Sprengart, & Porter, 1998; Wang et al., 1998). Taken together, it seems likely that a positive correlation exists between the accumulation of spectrin-breakdown products and activation of caspase-3; that is, α-spectrin cleavage is temporally related to caspase-3 activation. It has been shown that simvastatin increases cytosolic free Ca^{2+} concentration inducing apoptosis by activating caspase-3 via the calcium-dependent protease calpain (Cheng et al., 2003). Furthermore, arsenic trioxide has been shown to trigger apoptosis of bone marrow mesenchymal stem cells by modulating intracellular Ca^{2+} activating the caspase-3 activity (Cai et al., 2010). It is evident from the Western blots of α-spectrin that elevated Ca^{2+} concentrations caused SBDP120 intensity values to be significantly higher than in the control (Fig. 1(C, D)), but we cannot account for the lack of effects of camptothecin and etoposide treatments on the 120 kDa degradation products of α-spectrin (Fig. 1(A, B)). The effects of the two drugs on meat during the ageing/tenderization period were different from the effects observed with the previously mentioned cell lines. These differences in the breakdown of α-spectrin by caspase-3 may result from the lack of oxygen and ATP in meat tissue compared with the living cell lines. Despite this, camptothecin, etoposide and Ca^{2+} were likely involved in mechanisms that regulate apoptosis leading to a variation of caspase-3 activity, which potentially may aid in meat tenderization.

In this investigation, camptothecin, etoposide and Ca^{2+} treatments have all been shown to stimulate the degradation of troponin-T to the 30 kDa troponin-T fragment during 7 days postmortem ageing and particularly during the first 3 days (Figs. 2, 3, and 4). Meanwhile

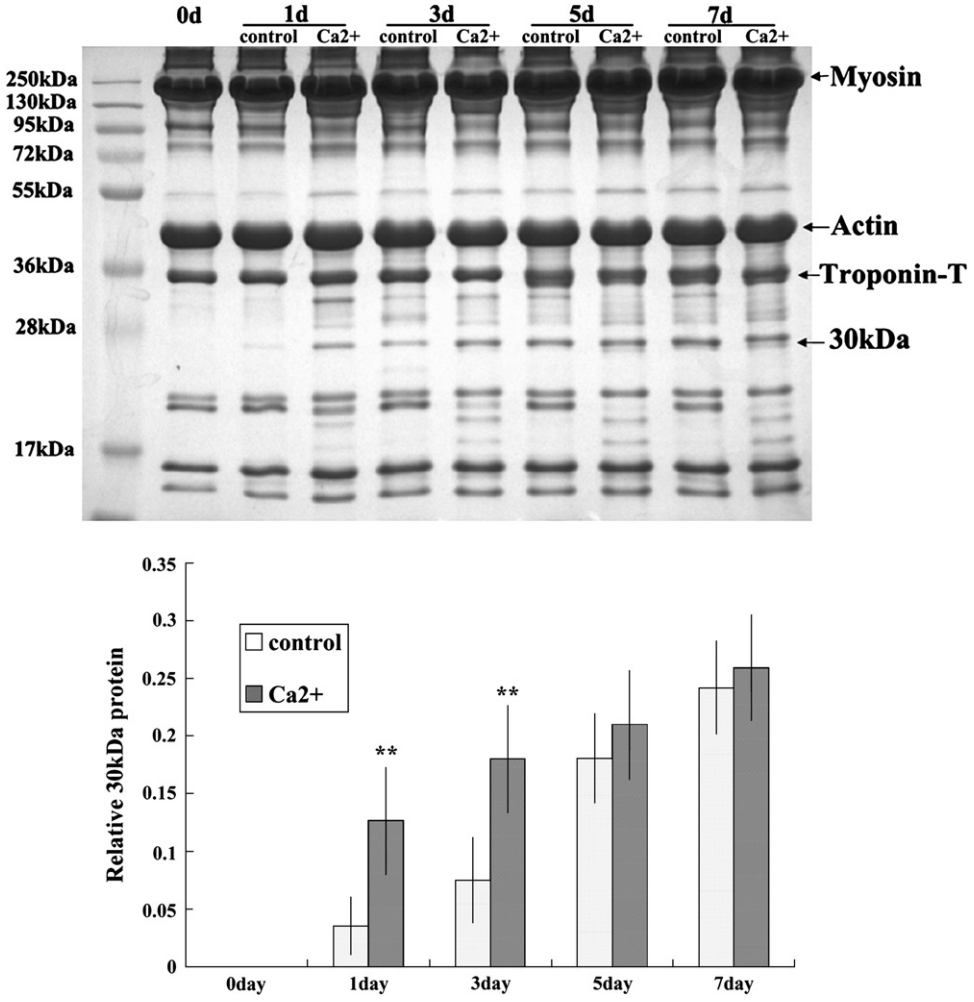

Fig. 4. Proteolysis of chicken breast muscle preparations in the presence (Ca^{2+}) and absence (control) of 0.05 M $CaCl_2$ when stored at 4 °C for 0, 1, 3, 5 and 7 days as shown by (A) SDS-PAGE of myofibrillar proteins (20 μg of protein per lane) together with the relative values (B) of the 30 kDa TnT fragment during 7 days of storage at 4 °C. Relative values were calculated as the blot densitometry of the actin in the same lane of each gel across the 7 days. Mean ± SD (n = 4). **: $p<0.01$ vs. control.

the results regarding the accumulation of 30 kDa troponin-T fragment confirmed those of previous studies involving ageing tenderization. Rather than having a direct structural impact on tenderness, degradation of troponin-T and accumulation of the 30 kDa fragment are often thought to be indicators of the overall postmortem proteolysis associated with ageing. The potential importance of the degradation of troponin-T on myofibril integrity was discussed by Hopkins and Thompson (2002), because of its association with thin filament proteins, which Taylor et al. (1995) suggested might influence the interaction of actin and myosin. At the same time, myofibril ultra-structures of the three treatments showed that the most prominent change was weakness of I-bands, which are contained in the thin filaments composed primarily of the actin (Fig. 5(D–L)). Troponin-T is a key protein for Ca^{2+}-sensitive molecular switching of muscle contraction (Ho et al., 1994). Supporting other investigations, the data showed that Ca^{2+} resulted in a dramatic increase in the troponin-T 30 kDa degradation product over the 7 day ageing period (Fig. 4). This supports the findings of Lawrence, Dikeman, Stephens, Obuz, and Davis (2004) who injected beef strip loins with 0 to 0.4 M $CaCl_2$ and found a greater degradation of troponin-T in the higher Ca^{2+}-treatment groups. Irrespective of the method of adding Ca^{2+}, $CaCl_2$ injection, marination or infusion of individual muscles or even carcasses, accelerated postmortem changes result in an improvement in tenderness (Koohmaraie, Babiker, Schroeder, Merkel, & Duston, 1988; Morgan, Miller, Mendez, Hale, & Savell, 1991; Wheeler, Koohmaraie, Lansdell, Siragusa, & Miller, 1993; Whipple & Koohmaraie, 1993). However, given that Ca^{2+} increases caspase-3 activities producing the distinct 120 kDa α-spectrin fragment, we propose there may be another route by which Ca^{2+} contributes to meat tenderness during ageing. That is by triggering apoptosis, and through a chain of events, leading to activation of caspase-3. Nevertheless, there are a large number of reports indicating that Ca^{2+} can play a central role in improving meat tenderness by its activation of calpain, the calcium-dependent neutral cysteine proteases. It has been suggested to be through the activation of μ-calpain (Koohmaraie, 1992), m-calpain (Boehm, Kendall, Thompson, & Goll, 1998), calpain-10 (Ilian, Bekhit, & Bickerstaffe, 2004a) and calpain-3 (Ilian, Bekhit, & Bickerstaffe, 2004b)). Although caspase-3, like calpain, is a cysteine protease it is not dependent on calcium, the results of this research convincingly shows that Ca^{2+} treatment could make caspase-3 more active and affect the substrate, α-spectrin (Table 1 and Fig. 1(C,D)) which could result in weakening of myofibrillar structures during postmortem ageing. Furthermore, the apoptosis chemicals camptothecin and etoposide also produced 30 kDa fragments of troponin-T in significantly greater amounts than in the control during the first 3 days of meat ageing (Figs. 2, 3), which indicates that these drugs contributed to myofibrillar proteolysis by inducing apoptosis and activating caspase-3. There is other evidence which suggests that caspase-3 makes an important contribution to the postmortem tenderization of meat (Huang et al., 2009; Kemp & Parr, 2008; Kemp et al., 2006). However, Underwood et al. (2008) found that caspase-3 activity was

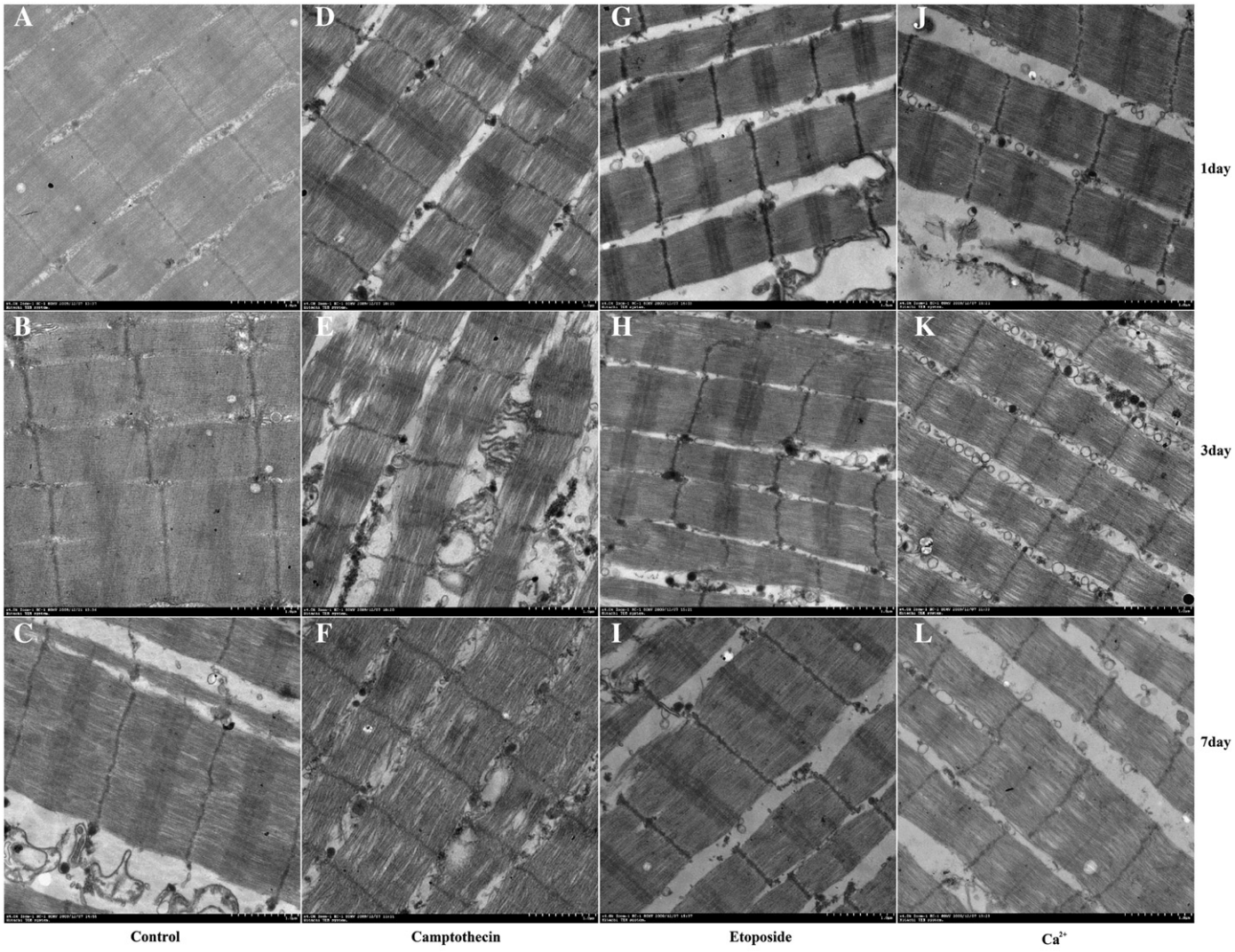

Fig. 5. Changes in the ultrastructure of longitudinal sections of muscle fibres from the chicken breast when stored for 1, 3 or 7 days post-mortem for Control (A, B, C), 40 mg/ml Camptothecin (D, E, F), 40 mg/ml Etoposide (G, H, I) and 0.05 M $CaCl_2$ (J, K, L).

not associated with Warner–Bratzler shear force, and they suggest that caspase-3 is not likely to be involved in postmortem tenderization of beef.

Electron micrographs of myofibrils showed that camptothecin, etoposide and Ca^{2+} treatments resulted in significant enlargement of the I-bands and length alteration of the A-bands, but the Z-lines were only slightly broken and largely remained intact until day 7 (Fig. 5). This agreed with reports which demonstrated the myofibrils were broken in the I-band region with little or no detectable degradation of Z-lines during this period (Taylor et al., 1995). Others have shown transverse disruption of sarcomeres in rabbit muscle at the N_2-line (nebulin and titin) which might indicate the major structural change responsible for meat tenderization (Mestre Prates, Garcia e Costa, Ribeiro, & Dias Correia, 2002). It has been demonstrated that the shrinkage of myosin and actin causes the I-bands to enlarge and the A-bands to shrink, producing gaps and discontinuity between nebulin and titin (Kong, Tang, Lin, & Rasco, 2008). Others have shown that in cattle, the spaces between myofibrils and the sarcolemma and also between the individual myofibrils increased during storage. It was also observed that the length of sarcomeres increased due to enlargement of I-bands and was accompanied by Z-line degradation (Kolczak, Pospiech, Palka, & Lacki, 2003). Following any one of the three treatments used here, the microstructures of the myofibrils changed in the following manner. In the first 3 days fading of the M-lines, shortening of A-bands, enlargement of I-bands and further disintegration of sarcomeres were observed which continued through to day 7 (Fig. 5). This is in agreement with Taylor et al. (1995) who showed that approximately 65 to 80% of all postmortem tenderization occurred during the first 3 or 4 days. In the present work, troponin-T cleavage also occurred during the first 3 days, and was stimulated by treatment with camptothecin, etoposide and Ca^{2+},mainly during the first 3 days, but continued until day 7 (Figs. 2, 3, 4, and 5). In addition on Ca^{2+} treatment, caspase-3 activities changed after day 3 and the 120 kDa degradation product (SBDP 120) of α-spectrin reached a high level by 3 days and did not change further through to day 7 (Table 1, Fig. 1(C,D)). The results strongly suggest that all three apoptosis inducers initiated chicken postmortem proteolysis faster by triggering an apoptosis cascade, and we attribute this to caspase-3 activation, which is an essential event for initiation of apoptosis. The dramatic disruptions observed for chicken myofibrils following camptothecin and Ca^{2+} treatments (Fig. 5) were similar to those reported for muscle in 0.2 M NaCl by pressurization at 200 MPa which resulted in the disappearance of the M-lines and depolymerization of each of the thin and thick filaments (Iwasaki, Noshiroya, Saitoh, Okano, & Yamamoto,

2006). Similar calcium-treatments caused the disappearance of M-lines and broadening of I bands after 24 h followed by disorganization of the structure of the I filaments at 168 h (Gerelt, Ikeuchi, Nishiumi, & Suzuki, 2002). Interestingly, electron micrographs of etoposide-treated muscle showed only a weak disintegration of myofibrils, as was also observed on treatment with camptothecin and Ca^{2+} (Fig. 5).

In summary, the findings provide further support for the role of caspase-3 in the tenderizing process during the early postmortem period. Clearly, further work needs to be done to separate and understand the roles of the two proteolytic systems, namely the calpain and caspase systems. In our view, the next investigations should concentrate on the associations between caspases and apoptosis during the early postmortem period.

Acknowledgments

This work was supported by the Earmarked Fund for Modern Agro-industry Technology Research System, China (Grant no: nycytx-42-G5-01) and Jiangsu Province Science Funding Project (Grant no: BK2009314). All authors read, commented on, and approved the final manuscript. The authors wish to thank Dr Ron Tume and Ammar for their careful revising of the paper and Feng Huang for his good advice.

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