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Influence of Epinephrine Infusion and Transportation Before Slaughter on Proteases Activity in Rabbit Muscles

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ABSTRACT- The objective of the present study was to determine if modifications would occur due to epinephrine perfusion and simulating stressful situations (2 µg/ kg/ min, an animal)and transportation,causing change of the values of extractable protein concentrations, SDS PAGE profiles and the calpain calpastatin complex in silver rabbit longissimus muscles resulting in consequent changes in meat tenderness. Approximately 60 standard rabbits, 70 days of age, were weighed and randomly assigned to epinephrine and transport treatments along with their controls at 3 times: immediately after slaughter (time 0), 24 hr and 7 days post mortem. The results showed there was a significant ($p<0.05$) lower value of extractable protein concentrations for treatments in control samples than the stress treated ones after 7 days of storage. Also, the comparison of SDS PAGE profiles at times 0 and seven days exhibited several differences due to the origin of the samples, especially around 30 KD, the intensity of the band is similar in all samples at time 0 while it decreases after seven days of storage in all samples except for those from the epinephrine treatment. On the contrary, after seven days of storage, the results indicated that dot blots against calpain were lower in the epinephrine samples while they were the highest in control treatments. Transport samples had an intermediate position. The results showed that epinephrine infusion and transport certainly modified muscular protein degradation. Therefore, variability in the rate of meat tenderization may arise as a result of stress induced activity or suppression of key proteolytic enzymes involved in myofibrillar protein turnover. Those alterations are due to modifications of the calpain calpastatin complex. The decrease in protein degradation was due to a decrease in calpain concentration.

Keywords: Calpain, Calpastatin, Epinephrine, Meat tenderness, Protein degradation, Transport

INTRODUCTION

Variation occurring in meat tenderness is due to genetics, biological and physiological differences, changes during slaughter, differences created during post-mortem storage, proteolytic activity or a combination of these factors (25 and 37). Lysosomal enzymes (cathepsins) have been hypothesized to play a role in postmortem proteolysis and meat tenderization because they degrade many of the same proteins that are degraded in post- mortem muscles.

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The calpain system also plays a role in meat tenderization. The calpain system is composed of several isoforms of the calcium dependent cysteine proteases (calpains) and their inhibitor (calpastatin) (11). The two best-characterized isoforms of calpains are mu-calpain and m-calpain. These isoforms are named in reference to the amount of calcium they need to be fully active in *in vitro* studies.

It is important to recognize that any of the stress factors during pre-slaughter handling can result in changes in the metabolites of muscles. These changes, in turn, are responsible for the differences in the ultimate properties of meat. The nature of the changes depends on such factors as the duration or severity of the stress, and the level of animal resistance such as, feed effect, feed withdrawal, acute heat stress and transportation. Hormones are one of the mediators of these stressful situations distributing the stress message in target tissues (brain, muscle, heart, liver, etc) (1, 6, 32 and 33).

Transportation stress has already been reported to reduce tenderness and increase lightness of chicken meat (5). Most pigs also show some evidence of fatigue and muscle glycogen depletion after long transportation, particularly under poor conditions leading to an increased incidence of DFD (dark firm and dry) meat (12 and 13).

It has been concluded that ß-adrenergic agonist (BAA) induced muscle hypertrophy is due to reduced protein degradation (40). In addition, feeding BAA decreases tenderness in lambs (7, 14, 20 and 27), cattle (30) and broilers (31) and tenderness is usually the result of postmortem proteolysis. Calpastatin activity at slaughter has previously been shown to increase in steers and sheep fed on a diet supplemented with a ß-adrenergic agents, such as cimaterol or clenbuterol (2, 15, 27, 36, 41 and 43). Such treatment results in an increased incidence of tough meat (8, 10, 27 and 42). Furthermore, calpastatin mRNA levels have also been shown to raise in steers fed with cimaterol (36) or in lambs fed with L.4644969 (18). The effects of ß-agonists on mu-calpain and m-calpain activity and mRNA have been less striking (2), suggesting that regulation of the system is likely to be exerted primarily through calpastatin. In the majority of these studies, administration of ß-adrenergic agonists for weeks resulted in an increased level of calpastatin and decreased calpain proteolytic potential (defined as m-calpain to calpastatin ratio) after slaughter and increased meat toughness after aging. The objective of the present study was to determine if such modifications would occurr after epinephrine perfusion as a simulating stressful situation (2 µg/ kg/ min, an animal) and transport on values of extractable protein concentrations, SDS-PAGE profiles and calpain-calpastatin complex in rabbit longissimus muscles.

MATERIALS AND METHODS

Experimental designs: Approximately 60 silver rabbits, 70 days of age, were weighed and randomly assigned to one of the following treatments: a) eight animals to be killed immediately after transferring out from the rearing cage (T animals), b) sixteen animals to be killed 30 min after transportation by car in a restricted cage (TC animals), c) fourteen animals to be killed immediately after 30 min of perfusion with epinephrine (2 µg/ kg/ min, an animal) (A animals) and d) fourteen animals to be killed after 30 min of perfusion with physiological serum (added ascorbic acid instead of epinephrine) (SP animals). Two groups were used as controls (T and SP)

and two were used as stressed ones (A and TC).

Muscle sampling was done 3 times: immediately after slaughter (time 0), 24 hr and 7 days postmortem. Sampling was done on *Longissimus lumborum* muscles. After harvesting, samples were immediately frozen in liquid nitrogen and stored at -80 ºC until analysis.

Extraction of Protein: Muscle samples were taken from the freezer and ground in liquid nitrogen. One gram of muscle powder was then homogenized in 20 ml of HIS buffer (50 mM K_2HPO_4, 24 mM KH_2PO_4, 1mM NaN_3, 1mM EDTA.Na_2 and 550 mM KCl) by an Ultra Turrax Mixer for 10 sec. The homogenate was shaken for 30 min (at 4°C) and then centrifuged at 11,000 × *g* for 30 min at 8ºC. The supernatant was decanted for all biological replicates, 2 technical replicates for each sample.

Protein Determination: Protein determination was done by BCA assay kit (Thermo Scientific) using bovine serum albumin (BSA) to provide a standard curve according to the manufacturer's instruction.

SDS-PAGE electrophoresis: A 40 µg protein samples were diluted in 100 µl loading buffer (2% SDS w/v, 3.5M ß-mercaptoethanol, 62 mM Tris-Cl pH 6.8, 10% Glycerol and a trace of bromophenol blue) and were treated at 95°C for 5 min. Discontinuous electrophoresis procedures were followed as described by Laemmli (28) using 10% polyacrylamide separating gels and 4% polyacrylamide stacking gels (1.0× mm 16× cm ×16 cm PROTEAN II xi CELL,Bio-Rad) in 35 mA current per 2 gel for 15 min which was then increased up to 70 mA for 5 hr and for all biological repeats and technical repeats.

Protein Staining: Gels were stained using Commassie Blue solution (0.05% coomassie blue R250, 45% ethanol and 5% acetic acid) overnight and were destained with a destaining solution (45% ethanol, 5% acide acetice). Gels were then scanned with EPSON EXPRESSION 10000 XL, Image Scanner III.

Dot blotting: A 20 µg protein sample was deposed on nitrocellulose membrane (Amersham) and dried before washing with Skimmed Milk-TBS-T buffer (SM-TBS-T, 20 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.05% Tween 20 and 3% skim milk) for 1 hr. The blot was incubated with the appropriate dilution of a primary antibody for 2 hr at room temperature (m-calpain 1/500, mu-calpain 1/2000, and calpastatin 1/5000, Affinity Bio Reagent) in SM-TBS-T buffer. The blot was washed 3 times for 5 min with TBS-T buffer and incubated with the secondary Horse Radish Peroxidase (HRP) conjugated antibody (1/1000 diluted concentration) in SM-TBS-T buffer for 2 hr at room temperature. The blot was washed for 15 min and 2 times for 5 min in TBS-T and then washed another time in TBS without Tween for 5 min. A chemiluminescent system was finally used to detect labeled protein spots as described by the supplier (Thermo Scientific). Volumes (staining intensity area) of spots were measured by Epson Expression 10000 XL, Image Scanner III scanner and used for statistical analysis after correction with an internal standard.

Western Blotting: SDS Gels, membranes and filter papers were briefly rinsed with transfer buffer (25 mM Tris, 192 mM Glycine and 20% methanol v/v) for 20 min. Protein bands were transferred onto nitrocellulose membranes using a Semi-Dry Blotting System EBU-4000(CBS SCIENTIFIC) at a constant voltage setting of 30V overnight at 4°C. Membranes were rinsed 2 times with TBS-T for 5 min and then incubated for 1 hr at room temperature in the blocking solution TBS-T containing 3% skimmed milk. Primary antibodies used for western blotting were monoclonal Anti-actin and Anti-mu-calpain (1/1000) diluted in TBS-T Skimmed-Milk for 2 h at room temperature. Membranes were then treated for the dot blot procedure and

protein bands were detected with chemiluminescent.

Statistical analysis: Data were analyzed using two-way analysis of variance with the GLM procedure of SAS 9.1. Mean values were compared using Student-Newman-Keuls'test.

RESULTS AND DISCUSSION

Values of protein ratio index including TC/T and A/SP for three times (t=0, 24h and 7days) are indicated in Figure 1. They show that there was a significant ($p<0.05$) lower value of protein concentration in T and SP as compared to A and TC samples after 7 days of storage. On the contrary, there were no significant differences between treatments at time 0 or 24 hr postmortem. This indicates that after 7 days of storage, proteins from "stressed" muscles were more extractible than those from the controls. Those results are in good accordance with previous works showing that increasing ß- adrenergic agonist and transport levels modifies protein turnover in the muscle by reducing proteolysis (3, 4, 9, 29, 38 and 41).

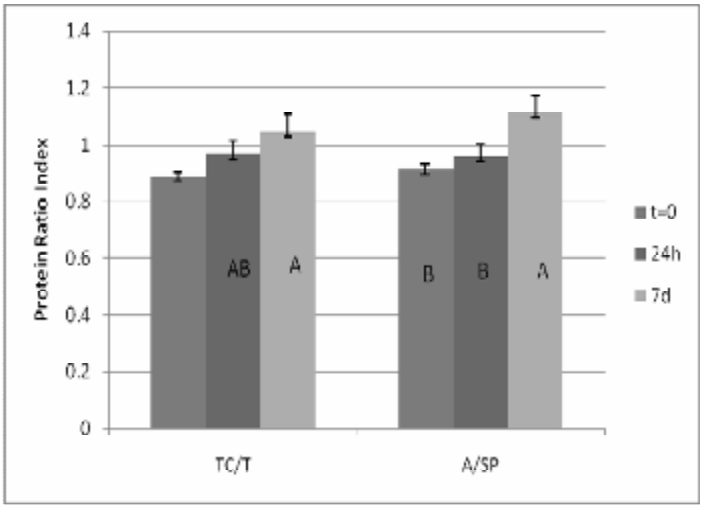

Fig. 1. Values of protein ratio index in treatments including A/ SP and TC/ T refer to animals that have been treated with epinephrine (2 µg/ kg/ min, animal), with ascorbic acid instead of epinephrine, transported by car in a restricted cage and killed immediately after transferring out from the rearing cage. T=0, 24h and 7d indicate times 0, 24h and 7 days postmortem, respectively. Means with different letters indicate a statistical difference ($p<0.05$) due to the time within a treatment

The comparisons of SDS-PAGE profiles at time 0 and day 7 exhibited several differences due to the origin of the samples. For example, we can see from Figure 2 that around 30 KD, the intensity of the band was similar for all samples at time 0 while it decreased after 7 days of storage for all samples except those from treatment A. On the other hand, the present of 30 KD polypeptide is related to degradation of troponin (39), nevertheless, the thickness of this band decreased after 7 days. In a number of studies T-troponin is determined as a regulatory protein without direct influence on meat tenderization but its post-mortem changes might indirectly improve the tenderness of meat by breaking the thin filaments and the interaction between thin and the thick filaments (16, 17 and 34).

We also showed that the intensity of the 30 KD band polypeptide decreased gradually from initial time to 7 days (Figure 3). Also, for molecular weights over 97 or 200 KD (see Figure 2) new bands were visible for 7 days samples but not for time 0 ones. These results suggest that the proteolytic degradation pattern of muscle proteins is modified in stress treated animals. Consistent with previous findings (8, 20, 26 and 43), BAA treatment and transport significantly reduced degradation of myofibrillar proteins during a 20-d postmortem storage. It is well documented that the following changes can be observed with SDS-PAGE analysis of longissimus myofibrils at different times postmortem (19, 21, 22, 23 and 35).

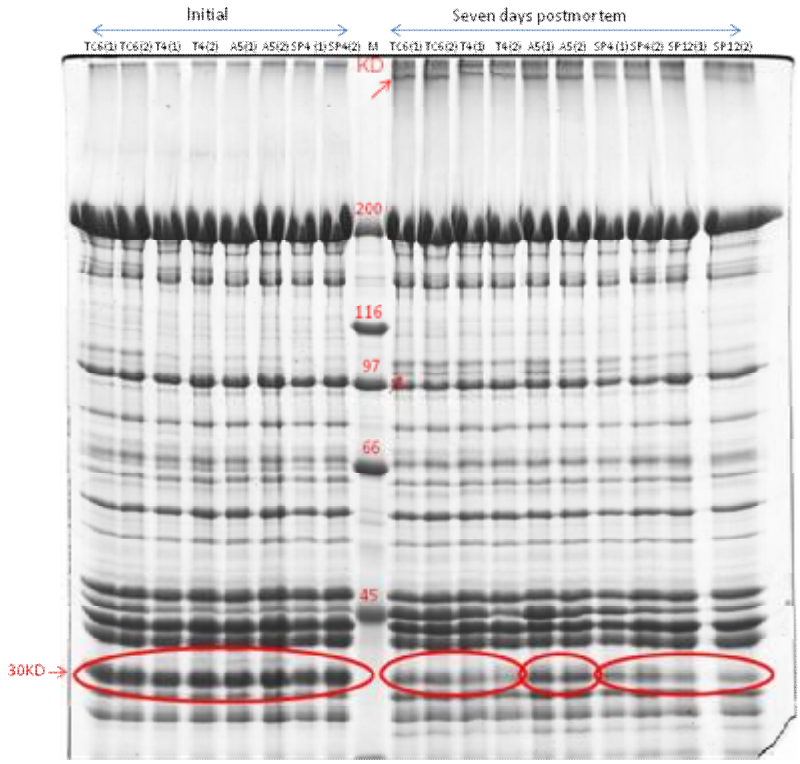

Fig. 2. Electrophoretically (SDS-PAGE) separated longissimus muscle proteins at two different times (initial time and 7 days postmortem) for the four treatments (A, SP, TC and T). Each treatment has two technical repeats

Protein dot blotting against m-calpain, mu-calpain and calpastatin was done for all treatments, (A, SP, TC and T) on proteins extracted at two different times (initial time and 7 days postmortem) (Figure 4).

Our data of dot blotting against three tested antibodies (Figure 5) showed that no significant differences were detected between the four treatments at initial time. On the contrary, after 7 days of storage, the results indicated that dot blots were always lower for A samples while they were highest for SP and T treatments. For the two calpain isoformes, TC samples had an intermediate position but they did not differ from Sp and T Samples for calpastatin. As shown in Figure 6, results for the m-calpain did not show any significant change from time 0 to day 7 despite the treatments. For mu-calpain, a significant decrease from time 0 to day 7 was observed only for the A samples. On the contrary, for SP and T samples, a significant increase was observed between time 0 and day 7. No significant change occurred in TC samples between time 0 and 7 for mu-calpain. For calpastatin, in all samples all the dot blot values increased from time 0 to day 7. Those alterations are due to modifications of calpain-calpastatin complex. Also, as our results show, decrease in

protein degradation is due to a decrease in calpain concentration. These results were similar to the findings of other studies (3, 20, 24 and 38).

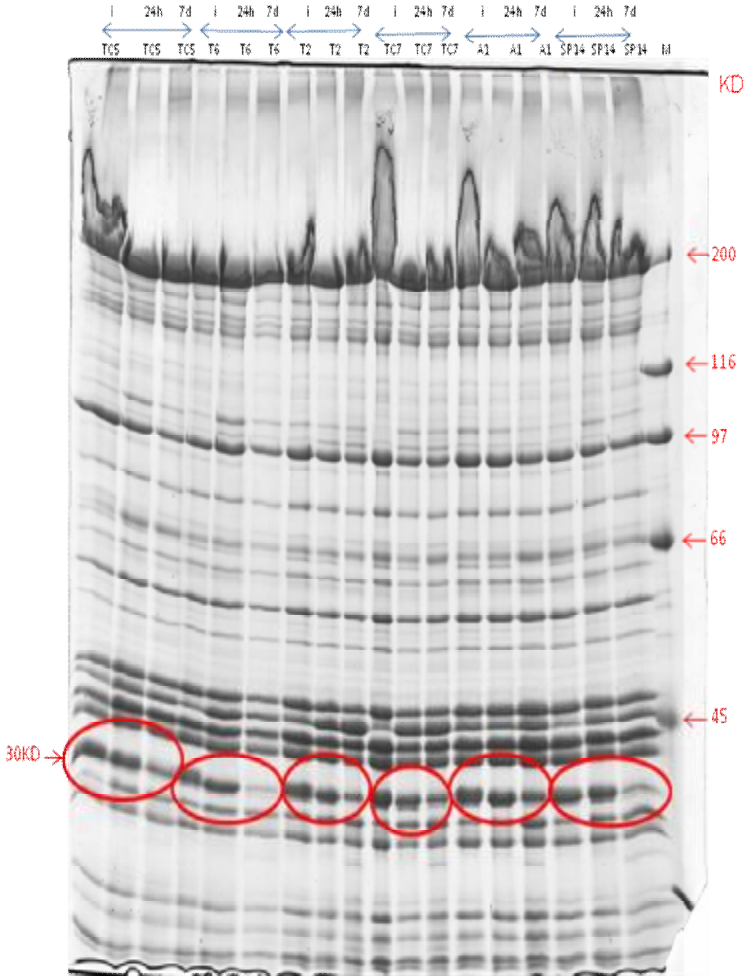

Fig. 3. Effect of postmortem storage on longissimus myofibrillar proteins from time 0, 24h and 7 days for A, SP, TC and T treatrments

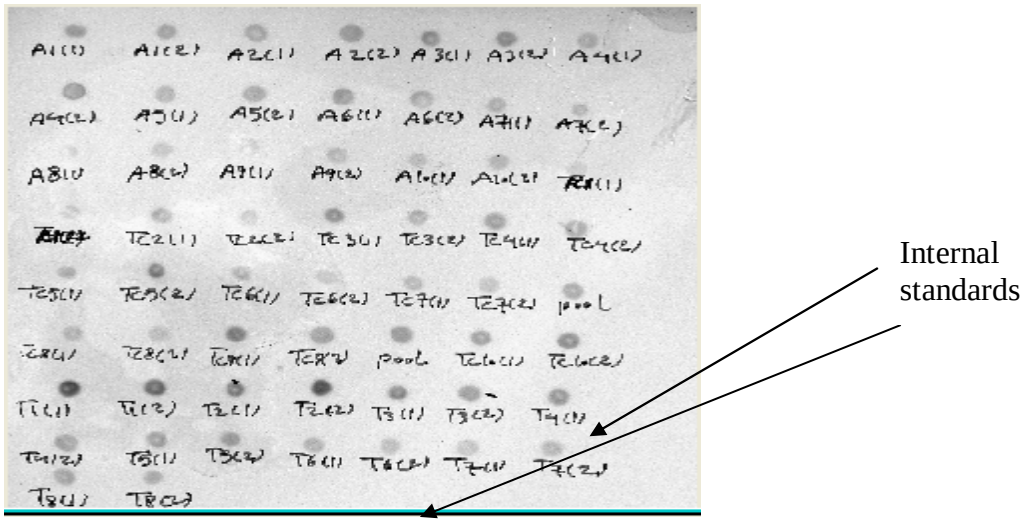

Fig. 4. Examples of dot blots against calpastatin for the four treatments (A, SP, TC and T) at two different times (initial time and 7 days postmortem)

Western Blotting analysis against mu-calpain appeared for the four treatments at the initial time but not for 7 days postmortem. It shows that those changes were only detected at the mu-calpain band at initial time. Whether stress treatments were present or not, there was no proteolysis detected through 7 days postmortem. Our results agree with Koohmarie et al (24) that the rate and extent of postmortem proteolysis determined by western blots reduced after 21 days postmortem.

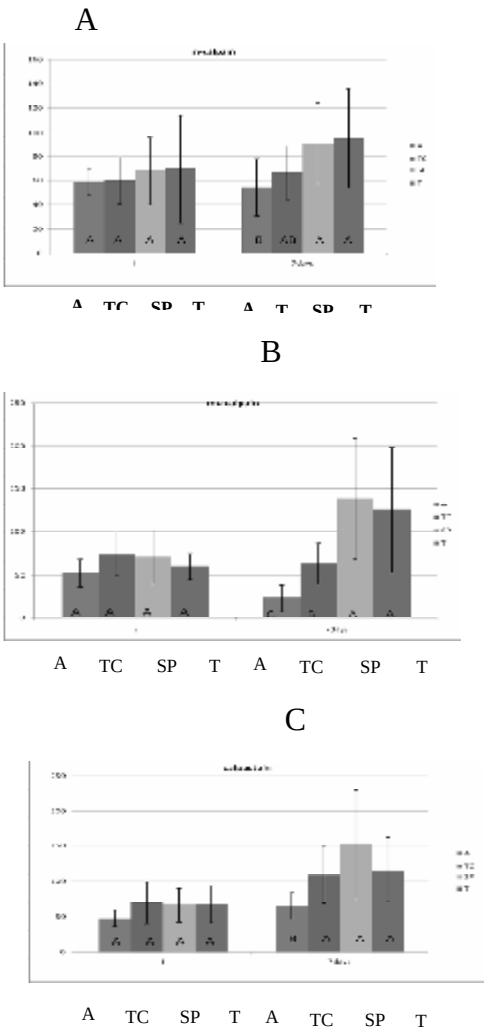

Fig. 5. Values of m-Calpain (A), mu-Calpain (B) and calpastatin (C) dot blots for A, SP, TC and T treatments at two different times (initial time and 7 days postmortem). Means with different letters indicate a statistical difference due to treatment for a given time ($p<0.01$). Error bars represent standard deviations. The number of A, TC, SP and T samples are 14, 16, 14 and 8 respectively

CONCLUSIONS

In conclusion, our results including extractable protein concentrations, SDS-PAGE profiles and dot blots showed that stress treatments (epinephrine and transportation) certainly modified muscular protein degradation. According to the findings, we can hypothesize that modifications of muscular proteolytic systems (i.e calpaïns) could alter postmortem proteolysis and result in meat tenderization. Hence, variability in the rate of meat tenderization may occur as a result of stress-induced or activity or

suppression of key proteolytic enzymes involved in myofibrillar protein turnover.

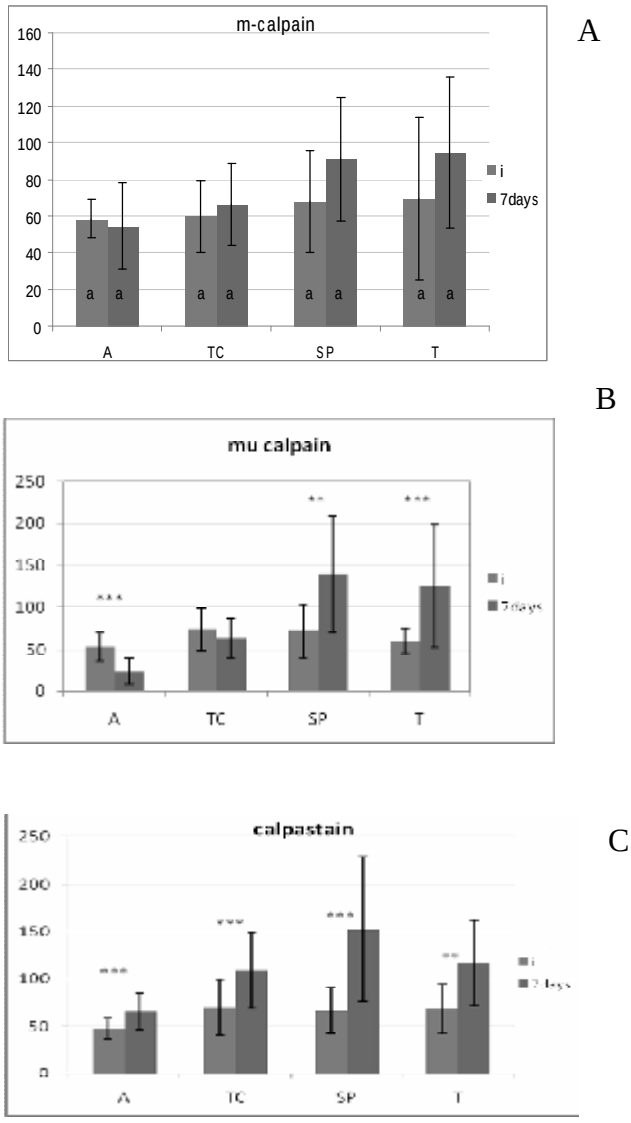

Fig. 6. Values of m-Calpain (A), mu-Calpain (B) and calpastatin (C) dot blots for A, SP, TC and T samples at two different times (initial time and 7 days postmortem). Means labeled with asterisks and different letters indicate statistical differences ($p<0.01$) due to storage for the different treatments. Error bars represent standard deviations. The number of A, TC, SP and T samples are 14, 16, 14 and 8 respectively

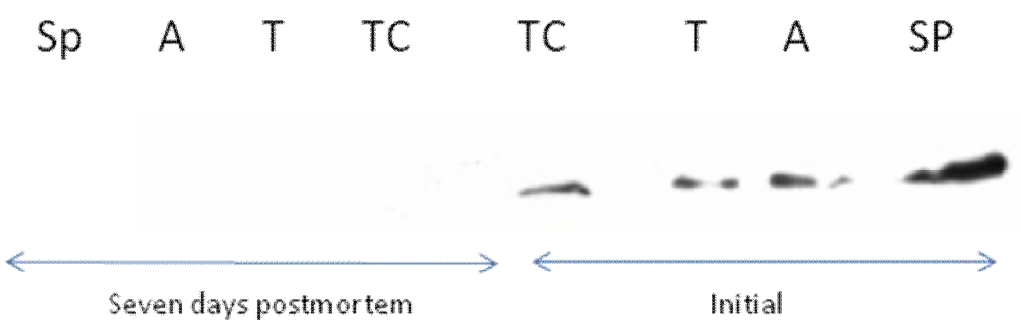

Fig. 7. Western blot using monoclonal mu-Calpain antibody for the four treatments (A, SP, TC and T) at two different times (initial time and 7 days postmortem)

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اثر تزریق اپی نفرین و تنش حمل و نقل قبل از کشتار روی فعالیت پروتئاز در ماهیچه خرگوش

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چکیده -هدف از این مطالعه تعیین تاثیر تنش تغییرات پس از تزریق اپی نفرین و تنش حمل و نقل برمیزان غلظت پروتئین استخراج شده از بافت های تحت تنش با نمونه های کنترل و همچنین مقایسه نتایج حاصل از الکتروفورز پروتئینها و کمپلکس کالپائین-کالپاستاتین در ماهیچه خرگوش سیلور است. حدود 60 خرگوش در سن 70 روزگی پس از وزن کشی بصورت تصادفی به دو تیمار اپی نفرین و حمل و نقل و کنترل های آنها در 3 زمان، صفر، 24 ساعت و 7روز پس از کشتار تقسیم شدند. نتایج نشان داد که کاهش معنی داری در میزان غلظت پروتئین استخراج شده در نمونه های کنترل نسبت به نمونه های تحت تنش 7روز پس از کشتار وجود داشت. همچنین مقایسات SDS-PAGE زمان صفر و ۷ روز پس از کشتار اختلافاتی را نشان دادند. خصوصا باند 30KD که در همه نمونه ها در زمان صفر مشابه بود. درحالیکه این غلظت 7 روز پس از کشتار در همه نمونه ها بجز تیمار اپی نفرین کاهش یافت. از طرف دیگر 7 روز پس از کشتار نتایج لکه گذاری بر علیه کالپائین در تیمار اپی نفرین پائین ترین و در نمونه های کنترل بالاترین را نشان دادند و تیمار حمل و نقل در موقعیت حد واسط بود. پس مقایسه میزان غلظت پروتئین استخراج شده از بافت های تحت استرس با نمونه های کنترل و همچنین مقایسه نتایج حاصل از الکتروفورز پروتئین های لکه گذاری و وسترن بلاتینگ برروی تجزیه پروتئینهای ماهیچه ای تاثیر معنی داری در تردی گوشت دارند. این تغییرپذیری در تردی گوشت بوسیله تنش ها به دلیل افزایش یا کاهش در آنزیم های کلیدی پروتئولتیک (از جمله تغییر در کمپلکس کالپائین-کالپاستاتین) درگیر درتغییر پروتئین میوفیبریلار است. همچنین ما در این تحقیق نشان دادیم که کاهش در میزان تجزیه پروتئین ناشی از کاهش در غلظت کالپائین می باشد.

وا ژه های کلیدی: ، اپی نفرین، تردی گوشت، حمل و نقل، کالپاستاتین، هضم پروتئین کالپائین

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