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INFLUENCE OF ELECTRICAL STIMULATION ON PROTEOLYSIS, CALPAINS ACTIVITY, FREE AMINO ACIDS CONTENT AND HISTOLOGICAL CHARACTERISTICS OF AGED EWE CARCASSES

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SUMMARY

One side from each of seven ewes carcasses, 5-6 years of age were electrically stimulated (ES) 25 Hz alternating current at high voltage (200 v) and low voltage (80 v) within 20 min. postmortem, the other side was non stimulated or control (NES). In both high or low voltage , had been an effect on the physiological fragmentation index (FI) and protein solubility (sarcoplasmic and myofibrillar proteins) than control (NES).As well as , ES side they had been an effect on calpain enzyme activity (CDP-I , CDP-II) and their inhibitor was in control the activity decreased gradually in low voltage and high voltage in descending orders . The results showed that ES treatment was more effective in release and increase of the content of free amino acid such as : Glutamic , Hydroxyproline and Alanine comparison to control.

Gel electrophoresis pattern of myofibril protein extraction from the Semitendinosus (ST) and Triceps Brachii (TB)in ES treatments demonstrate distinct hydrolysis of most meat proteins . Transmission electron microscopy results showed greater degree of ultrastructural in myofibril , disordered array of sarcomeres fragmented and loss of Z-line, leaving gaps in the middle of the sarcomere.

It can be concluded from this study that electrical stimulation had positive significant influence on protein solubility, calpains enzyme activity, free amino acid content . and histological characteristics and lead to improve meat quality characteristics of aged ewe carcasses.

Keywords: *Electrical Stimulation ,protein solubility, calpains enzyme, free amino acid, aged ewe carcasses*

INTRODUCTION

Electrical stimulation involves passing an electric current through the body or carcass of freshly slaughtered animals. This electric current causes the muscles to contract ,increasing the rate of glycolysis and resulting in an immediate fall in muscle pH and subsequent early rigor mortis

development (Hwang et al.,2003).Other potential effects of stimulation on post-mortem muscle are described as either physical disruption of myofibrillar matrix (Ho et al.,1997) or acceleration of proteolysis (Uytterhaegen et al.,1992) . Increase the content of free amino acids during storage, the primary reason for this accumulation of free amino acids in meat to be the action of protease, peptidase or both (Mikami et al., 1994 and Al-Rubeii, 2004). Myofibrillar proteolysis can be attributed to endogenous proteinase activity. Currently, two characterized proteolytic systems are known to hydrolyzed myofibrillar proteins, the Ca^{+2} _dependent (calpains_ I and Π) and lysosomal acidic proteinase systems (cathepsins B, D, H and L)(Dransfield et al.1992). ES accelerates the activation of calpains are responsible for myofibrillar protein degradation proteolysis and tenderisation of meat postmortem (Hopkin and Thompson, 2001). Dutson et al. (1980) and Lee et al. (2000) they have showed that lysosomal membranes were disrupted and that activity of free lysosomal enzyme increased after ES. Many studies have been reported about changes in myofibrillar proteins after ES, but there is little data on protein solubility (sarcoplasma and myofibrillar protein) and free amino acids content of aged ewe carcasses. The objective of this study is to investigate the effects of electrical stimulation on protein solubility, calpains activity, free amino acids content and histological characteristics in aged ewes carcasses.

MATERIALS AND METHODS

Animals and Stimulation

Seven local Awassi ewes approximately 4 to 6 years old were slaughtered dressed and divided into two sides for experimental treatment in the department of Animal production at the College of Agriculture, Abu-Ghraib. Each side was transferred into a temperature controlled room at 16 ± 2 C^{o} and ES were applied. One side from each carcass was stimulated within 20 min. postmortem. Using high voltage (200 v) (4 sides) and low voltage (80 v) (3 sides) with frequency 25 Hz, alternating current 3A with a square wave pulse for 2 min. on for 1 sec. and off for 1 sec. according to (2), while 7 sides were kept as non stimulated (NES) or control group. After electrical stimulation the sides were kept at the same temperature until the pH of the muscles reached 6.0, then the sides were subjected to a chilling temperature at 2 °C for 24 hr postmortem. Three muscles namely the SM (Semimembranosus), ST (Semitendino-sus) and (TB) Triceps Brachii were taken to be measured and analysed for various parameters as described below:

Fragmentation Index (FI)

Samples were excised from the SM.ST and TB muscles at 24 h postmortem .FI were determined according to the procedures of Davis et al. (1980).

Protein Solubility

The solubility of protein fractions (sarcoplasma and myofibrillar protein) was determined according to procedure of DenHertog-Meischke et al. (1997).

Preparation and Assay of Calpains (I , II) and their Inhibitor Activities

Samples (25 g) of the SM,ST and TB muscles were taken at 24 hr. postmortem , and prepared according to (Koohmaraie et al.,1989) and the activities of calpains I and II were determined using casein as a substrate as described by (Koohmaraie et al.,1989). One unit of calpain activity was defined as the amount of enzyme required to catalyze an increase of 1.0 absorbance unit at 278 nm. In 60 min. at 25 °C. The activity of calpain – inhibitor were determined according to (Wheeler and Koohmaraie,1991). One unit of inhibitory activity was defined as the amount of the inhibitor that inhibits one unit of calpain – II activity .

Amino acid analysis by reverse–phase High Performance Liquid Chromatography (HPLC)

Samples (15 g) were taken at 24 hr. postmortem from ST and TB muscles. Samples were prepared for amino acid extraction according to (Mikami et al.,1994) . Using the method of (Heinrikson and Meredith,1984) to prepare the derivatization of amino acids by using phenylisocyanate. Using HPLC system (Shimadzu LC-6A) supplied by the microprocessor controlled S11 – 6A unit. 10 µL of the derivative solution was injected on to Grafted Ultra Sphere. ODS (c-18) column (5 µm particle size , 250 x 4.6 mm I.D.) was employed . The elution gradients were conducted at flow with wavelength detector at 254 nm.

Myofibril Isolation and SDS_PAGE

Myofibrils from ST and TB muscles were isolation according to the methods of Olson et al.(1976).protein concentration were determined by biuret procedure. Electrophoretic (SDS_PAGE) procedures of Laemmli (1970)and Greaser et al.(1981)were followed with 75ug of protein loaded per well. Myofibrillar proteins were separated using a discontinuous 6 to 12.5% acrylamide gradient.

Preparation for Transmission Electron Microscopy

Ultrastructural changes in control and ES muscle have been analysed by observation of longitudinal section of muscle in transmission electron microscopy after ES treatment .Srtrips of ST muscle approximately 4mm wide,40mm long and 2 mm deep were fixed by immersion in 2.5%glutaraldehyde , fixation , dehydration , embedding ,sectioning and staining were used according to the methods of Bancroft and Stevens(1977) and Tarnawski et al.(1989).Then observed with a Philips EM 400 electron microscope under an acceleration voltage of 60 KV at (X20000).

Statistical Analysis

Data was statistically analyzed using General Linear Model (GLM) procedure by (SAS,2001) where electrical stimulation as a main effect. Duncan's multiple range test was used to determine the significance of differences between treatments means and to determine the significance of differences between muscles in the treatment.

RESULTS AND DISCUSSION

Fragmentation Index (FI) of muscle was related to overall carcass physiological maturity and lean texture .The ES treatments (80,200 V)

effects FI were significant (P<0.05) (Table 1).The ES muscles showed lower FI and higher filtrate volume in comparison with NES treatment. These observation agree with Al-Rubeii (2003) in aged female goat who found that stimulated muscles had exhibited greater degradation in myofibrils ,it is conceivable that ES has produced physical disruption of muscle tissue and lead to weakening of longitudinal structure of myofibrillar sarcomeres and the integrity of muscle (Lee et al.,2000).Also, in ST muscle both FI decreased (P<0.05) and filtrate volume increased (P<0.05) than the SM and TB muscles. These results support the findings of Yanar et al.(1999).

The protein solubility of fraction (sarcoplasma and myofibrillar protein) for SM, ST and TB muscles from electrical stimulated sides are shown in Table (2).The data shows that ES had significant effect (P<0.05) on the solubility of sarcoplasmic and myofibrillar protein and the stimulated muscles exhibited greater (P<0.05) solubility than NES muscles. These results were supported by results of Hopkins and Thompson (2001) in ovine muscles and Al-Rubeii (2003) in aged female goat meat and that probably was due to release of lysosomes enzymes as results the increased rate of pH fall after stimulation. Furthermore, ES accelerating the activation of calapin system (Dransfield et al.,1992).The ST muscle seams to be more proteolytically active than SM and TB muscles .Similar results were reported by Kerth et al.(1999) who found that myofibrillar fragmentation index in ST muscle 40.9 while in SM muscle 36.2 therefore the ST was tender than the SM .

Presented in Table (3) are comparisons of activation of calpain – (I , II) and their inhibitor for SM,ST and TB from electrical stimulated and non stimulated muscles .ES positively (P<0.05) on activities of calpains, ES muscles showed faster decrease of calpain –I , II and their inhibitor activities than did NES .ES accelerated activation of calpain system during the early postmortem stage and reduced the remaining enzyme activity (Lee et al.,2000), may be due to ES immediately after slaughter accelerated postmortem glycolysis with concomitant rapid pH decline with affects proteolytic (Hwang et al.,2003) as well as, the increased calpain activity may be due to depolarization of the cell membrane (Morton and Newbold,1982) that causes a release of calcium into the cell ,which then activates the Calcium-Dependent Proteases (calpain) in early postmortem period may be due to reduced remaining enzyme activity which coincided with tenderization involving postmortem proteolysis of myofibrillar /cytoskeletal protein (Lee et al.,2000) .The results in this study support those reported by Dransfield et al.(1992) and Al-Rubeii (2004) . The higher total activity residue in calpain enzyme (I , II) and their inhibitor of TB was found in a contrary to that in ST , and ascribed to higher calpain –II and inhibitors level in slow-twitch red muscles than in fast – twitch white muscles , therefore , degradation of the myofibrillar structure has been shown to occur more rapidly in fast twitch white fibers than in slow – twitch red fibers (Ouali ,1990).

Free amino acids composition for ST and TB are shown in Table (4). Almost all free amino acids increased

Table (1): Means values and standard errors (SE)for effects of electrical stimulation fragmentation measures in Semimembranosus (SM), ST (Semiten dinosus) and Triceps Brachii (TB).

Treatment	SM		ST		TB	
	FI%	Vol/Fil. ml	FI%	Vol/Fil. ml	FI%	Vol/Fil. ml
NES	A 290 ab	C 40.00 a	A 280 b	B 41.00 a	A 320 a	C 40.00 a
ES/80v.	B 205 a	B 44.00 b	B 190 b	A 45.00 a	B 215 a	B 44.00 b
ES/200v.	C 150 b	A 47.00 ab	C 148 c	A 48.00 a	C 160 a	A 46.00 b
Means ±	215.00 ±	43.66 ±	206.95 ±	44.66 ±	231.66 ±	43.33 ±
SEM	3.55	1.25	2.75	1.30	2.58	1.70

SEM = Standard error of means . A , BC means in the same column for significant differences between NES and ES ($P < 0.05$) , a , b : means in the same row for significant difference between SM.ST and TB for each treatment ($P < 0.05$).

Table (2): Protein solubility (mg/g meat) of(sarcoplasmic and myofibril) in Semimembranosus (SM),ST(Semitendinosus) and Triceps Brachii (TB). muscles as affected by electrical stimulation ± SEM [1].

Treatment	SM		ST		TB	
	Sarcoplasmic	Myofibril	Sarcoplasmic	Myofibril	Sarcoplasmic	Myofibril
NES	B 52.90 ab	C 63.55a	B 53.30 a	C 64.20 a	B 52.10 b	C 61.39 b
ES/80v.	A 54.80 a	B 65.93a	A 55.70 a	B 66.12 a	A 53.59 b	B 62.55 b
ES/200v.	A 54.70 b	A 67.11a	A 55.40 a	A 67.67 a	A 53.40 c	A 63.97 b
Means ± SEM	54.15 ± 2.42	65.55 ±1.29	54.80 ± 2.31	65.99 ± 1.55	53.03 ± 2.10	62.63 ± 1.80

SEM = Standard error of means . A , BC means in the same column for significant differences between NES and ES ($P < 0.05$) , a , b : means in the same row for significant difference between SM.ST and TB for each treatment ($P < 0.05$).

Table (3). Comparison of enzyme activity (unit / 25 g muscle) between treatment for Semimembranosus (SM),ST(Semitendinosus) and Triceps Brachii (TB) ± SEM[1].

Treatmen	SM			ST			TB		
	CDP-I (2)	CDP-II (2)	Inhibitor (3)	CDP-I	CDP-II	Inhibitor	CDP-I	CDP-II	Inhibitor
NES	A 40.50b	A 62.00b	A 80.00a	A 30.0(	A 58.20c	A 53.80b	A 51.00a	A 72.00a	A 88.40a
ES/80v	B 23.50b	B 48.00b	B 50.50b	B 24.1(	B 47.00b	B 46.00c	B 30.30a	B 54.00a	B 67.20a
ES/200v.	C 22.00b	C 37.60c	C 45.00b	C 21.0(	C 41.10b	C 40.00c	C 28.00a	C 50.50a	C 57.00a
Means	28.66	49.20	58.50	25.03	48.76	46.60	36.43	58.83	70.86
±SEM	±2.50	±2.73	±1.30	±3.10	±3.51	±2.45	±1.80	±1.64	±3.22

1. SEM = Standard error of means . A , BC means in the same column for significant differences between NES and ES ($P < 0.05$) , a , b : means in the same row for significant difference between SM,ST and TB for each treatment ($P < 0.05$).

2. Calpain –I , Calpain –II = Total activity / 25 g muscle (caseinolytic activity).

3. Inhibitor of calpain – I and II , A278/25 g muscle (inhibition of casein hydrolysis by calpain –II).

Table (4). Free amino acid composition (mg/100 g meat) for Semitendinosus (ST) and Triceps Brachii (TB) muscles as affected by electrical stimulation ± SEM.

F.A.A.	ST				TB			
	NES	ES/80 v	ES/200 v	SEM	NES	ES/80 v	ES/200 v	SEM
ASP	B1.60a	A1.78a	A1.82a	1.73±0.01	B1.49a	A1.65a	A1.74a	1.62±0.03
Glu	B15.5a	A26.3a	A27.05a	23.05±1.15	B15.3b	A25.60b	A24.80b	22.03±1.20
Ser	B4.20a	A4.40a	A4.52a	4.37±0.08	A4.03a	A4.18a	A4.28a	4.16±0.04
Hyp	C1.51a	B3.28a	A4.67a	3.15±0.45	C1.30b	B2.80b	A4.30b	2.80±0.51
Gly	B7.00a	A7.32a	A7.39a	7.23±0.19	B6.15b	AB6.35b	A6.73b	6.41±0.15
His	C5.40a	B5.90a	A6.50a	5.93±0.23	C5.27a	B5.70a	A6.10b	5.69±0.25
Thr	B3.40a	AB3.5a	A3.60a	3.50±0.52	B3.22a	AB3.39a	A3.55a	3.38±0.39
Ala	C34.1a	B35.7a	A36.45a	35.42±0.60	C33.4a	B34.90b	A35.65a	34.65±0.55
Arg	B4.70a	A4.91a	A5.19a	4.93±0.20	C3.90b	B4.40b	A4.82a	4.37±0.13
Pro	B3.00a	AB3.2a	A3.40a	3.19±0.05	B2.50b	A2.99a	A3.15a	2.88±0.11
Tyr	B2.19a	A2.35a	A2.49a	2.34±0.10	B2.08a	A2.30a	A2.39a	2.25±0.12
Val	C3.65a	B3.90a	A4.10a	3.88±0.11	B3.55a	AB3.66a	A3.90a	3.70±0.06
Met	C1.31a	B1.64a	A1.75a	1.56±0.08	B1.20a	A1.43a	A1.52a	1.38±0.13
Cys	A1.25a	A1.31a	A1.35a	1.30±0.12	A0.82b	A0.90b	A0.94b	0.88±0.11
Ile	B2.45a	A2.75a	A2.89a	2.69±0.11	B2.20b	AB2.35b	A2.41b	2.32±0.12
Leu	B4.32a	AB4.6a	A4.75a	4.55±0.32	B3.90b	A4.15b	A4.28b	4.11±0.35
Phe	B2.67a	A2.90a	A2.97a	2.84±0.28	B2.51a	A2.75a	A2.82a	2.69±0.20
Lys	B4.22a	A4.64a	A4.70a	4.52±0.20	B3.75b	AB3.85a	A4.02b	3.87±0.11
Total	C102.4a	B120.3a	A125.5a	116.145.10±	C96.68b	B113.3b	A117.4b	109.14±4.55

SEM = Standard error of means. A , B , C means in the same row for significant difference between NES and ES ($P < 0.05$) . a , b means in the same row for significant difference between LD and BF for each treatment ($P < 0.05$).

after ES. In this study the mean values for Glutamic (Glu), Hydroxyproline (Hyp), Histidine (His) and Alanine (Ala) are present significant differences ($P<0.05$) among the NES and ES treatments . The ES/200 and 80 v. treatments have the highest ($P<0.05$)total amount of free amino acids in ST(125.59 and 120.36 mg/100 meat) and TB (117.40 and 113.35 mg/100meat) muscles respectively ,on the other hand the NES treatment recorded the lowest total amount of free amino acids in ST(102.48 mg/100g meat) and TB(96.86 mg/100g meat muscles).The ST muscle had higher total amount of free amino acids in all treatments. The increase in free amino acids content after ES treatment has been thought to be due to the action of proteolytic enzyme located in cytoplasm (calpain) or release partly from lysosomes (amino peptidase and cathepsins).As well as , Estreatments increases the activity transaminase was about 20% greater than the NES that contributes to accumulation of some amino acids such as Glu (Sekikawa et al.,1999).Similar results were reported by Mikami et al.(1994) in beef muscle and Al-Rubeii(2004) in aged ewe meat.

The results of SDS-PAGE (Figure 1(A and B)) showed that distinct hydrolysis of most meat proteins in ES treatments than NES, may be due to ES caused a faster degradation of protein such as titin and troponin –T in muscle (Ho et al.,1996).Furthermore, calpains catalyze the degradation of cytoskeletal protein such as titin, nebulin and desmin (Taylor et al., 1995).Any conditions that enhance postmortem activity of calpain system would results in more degradation of these cytoskeletal proteins. Thus, results of this study indicate that rapid degradation of titin was caused by ES induced activated calpain system. It seemed that the ST muscle responded more to ES proteolysis than the TB muscle in all treatments. This results were supported by results of Rhee et al.(2000) in beef muscles and Lee et al.(2000)in ovine muscles who showed that stimulation dose increase the rate of postmortem proteolysis.

Histological images of stimulated muscle showed that causing physical alteration either through it is effects on the appearance of contractile bands containing predominantly stretched ,ill defined and disrupted sarcomeres (Figure 2, B and C)as well as the fragmentation of ultrastructural in myofibrils causing to disordered array of sarcomeres fragmented loss of Z-line and leaving a gaps in the middle of sarcomere. These disturbance could account for an accelerated proteolysis. There is potentially a powerful association between physical disruption of nyofibrillar complex and increases in tenderness (Ho et al.,1996) therefore, the increase in tenderness due to distruption was a plansible finding as the historical reason for use of ES was to prevent cold shortening, Savell et al.(1978) first reported the contraction bands and slightly stretched sarcomeres in ES beef muscle .Yanar(2000) and Al-Rubeii (2003) also proposed that ES resulted in muscle tissue disruption. The results are in agreement with findings of our present study.

CONCLUSIONS

Data from the present study suggest that ES can be used to enhance the

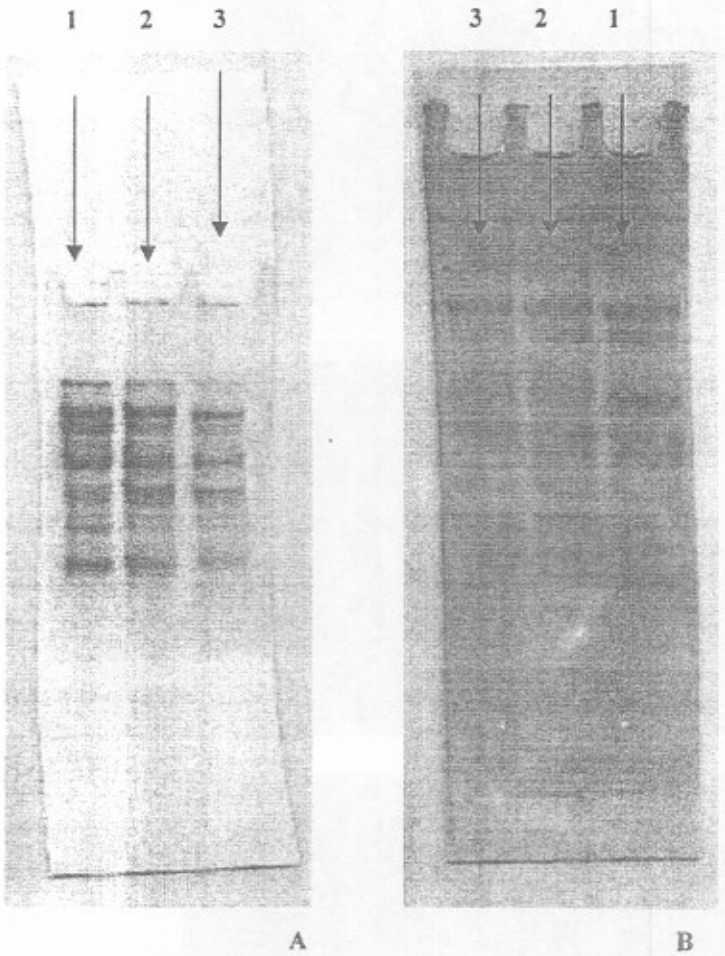

Figure (1): (A and B),The SDS-PAGE (6 to 12.5%) of purified myofibril is taken from Semitendinosus (ST) (A) and Triceps Brachii (TB) (B) muscles at 1 d postmortem .NES (1), ES 80 v. (2) and ES 200 v. (3)

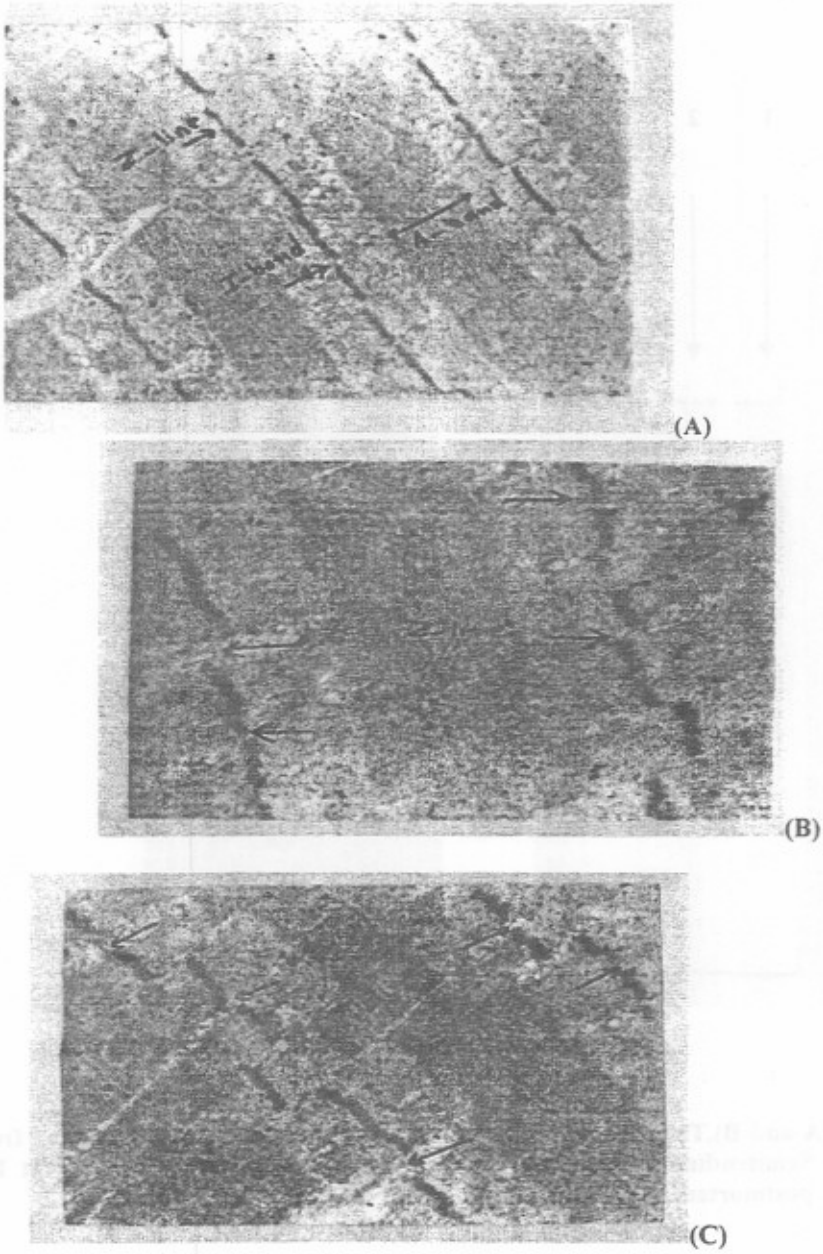

Figure (2). Electron microscope (x20000)images for characteristics of contracture bands in electrically stimulated Semitendinosus (ST) compared to control. NES (A),ES 80 v.(B) and ES 200 v.(C) .

protein solubility and ES can promote the activity of endogenous proteolytic enzymes including accelerate activation of calpains as well as , ES increases the content of free amino acid in meat may to enhance the flavor meat electrical stimulation and contribute of physical disruption to improvements in tenderness of aged ewe meat.

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تأثير التحفيز الكهربائي في ذائبية البروتين وفعالية الكالبينات ومحتوى الأحماض الأمينية الحرة والصفات النسيجية للحوم ذبائح النعاج المسنة

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استهدفت التجربة دراسة تأثير التحفيز الكهربائي في ذائبية البروتين وفعالية الإنزيمات الداخلية (الكالبين) ومحتوى الأحماض الأمينية الحرة والصفات النسيجية للحوم ذبائح النعاج المسنة . استعمل في التجربة ٧ نعاج مسنة بعمر ٥-٦ سنوات ، ذبحت النعاج وتمت سلاختها وتجويفها وشطرها الى نصفين . بعدها وزعت أنصاف الذبائح عشوائياً الى ثلاث معاملات هي معاملة السيطرة (٧ أنصاف) ، معاملة التحفيز الكهربائي (٨٠ فولت ، تردد 25 Hz لمدة دقيقتين) (٣ أنصاف) ، معاملة التحفيز الكهربائي (٢٠٠ فولت تردد 25 Hz لمدة دقيقتين) (٤ أنصاف) . وقد لوحظ أن كلا معاملتي التحفيز الكهربائي (٨٠ و ٢٠٠ فولت) أظهرت تأثيراً معنوياً في قيمة دليل التكسر الفسيولوجي (FI)و ذائبية البروتين (الساركوبلازما والمايوفيبرل) مقارنة مع معاملة السيطرة ، فضلاً عن ذلك تبين من النتائج وجود تأثير معنوي للتحفيز الكهربائي في فعالية إنزيم الكالبين (I ، II) ومثبطهما اذ انخفضت الفعالية المتبقية تدريجياً في معاملة التحفيز الكهربائي ٨٠ فولت ثم ٢٠٠ فولت عن معاملة السيطرة. وكذلك وجد تأثير معنوي للتحفيز الكهربائي في الزيادة المتحققة في محتوى الأحماض الأمينية الحرة (حامض الكلوتاميك ، الهيدروكسي برولين والإعلانين) مقارنة مع معاملة السيطرة. و أوضحت نتائج الترحيل الكهربائي الهلامي لمستخلص بروتينات المايوفيبرل في العضلات ST,TB حصول تحلل بروتيني واضح في اغلب بروتينات اللحم بتأثير معاملتي التحفيز الكهربائي أكدت ذلك نتائج الفحوص النسيجية حدوث تغيرات كبيرة في التركيب الدقيق لليف العضلي وعدم انتظام تركيب الساركومير وظهور تحطم واختفاء Z-line ووجود فجوات في وسط الساركومير بتأثير معاملتي التحفيز الكهربائي مقارنة مع معاملة لسيطرة .

يستنتج من الدراسة بأن هناك تأثيراً معنوياً للتحفيز الكهربائي في ذائبية البروتين وفعالية الكالبينات ومحتوى الأحماض الأمينية الحرة والصفات النسيجية مما يؤدي الى تحسين الصفات النوعية للحوم المنتجة من ذبائح النعاج المسنة.

الكلمات الدالة: *التحفيز الكهربائي،ذائبية البروتين،فعالية الكالبينات ،الأحماض الأمينية ،ذبائح النعاج المسنة .*