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The Molecular Mechanism of ES Improved Beef Tenderness Identified by Two-Dimensional Protein Electrophoresis

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Abstract: Differential proteomics in electrical stimulated M. longissimus of Chinese yellow crossbred bulls (ES: 42 V, 50 Hz, 0.7 A for 40 sec) were carried out using two-dimensional electrophoresis technology. Twelve protein spots had reduced expression at 1 and 3 days post Electrical Stimulation (ES) of samples which were divided into nine categories: desmin, troponin T alpha isoform, myosin binding protein H, creatine kinase (two), triosephosphateisomerase (two), peroxiredoxin-6 (two), phosphatidylethanolamine-binding protein, histone H3.3-like isoform 2, methyltransferase. Biological function analysis of these proteins indicated ES affected tenderness via four pathways: glycolytic metabolic pathway, calpains pathway, lysosomal pathway and oxidative stress pathway. Correspondingly, we concluded that ES could accelerate the decline of pH and the release of lysosomal enzymes, activate the calpain system and accelerate oxidation and apoptosis. All these combined effects accelerate the meat tenderization process. Results provide a relatively comprehensive understanding of the influences that ES exerted on beef tenderness improvement from the perspective of proteins changing. Furthermore, the results indicate that more attention should be paid on the contribution of lysosomal to beef tenderness and the effects of oxidative stress on beef tenderness point out the further research direction and related experiments have been designed to verify such specific mechanism.

Key words: Electrical stimulation, differential proteomics, chinese yellow crossbred bulls, perspective, accelerate

INTRODUCTION

Tenderness is the most important beef quality attribute which determines the overall consumer satisfaction (Lomiwes *et al*., 2014; Ouali *et al*., 2013; Marino *et al*., 2013). Beef tenderness is not only affected by physiological impacts such as species, feeding and cattle age but also by postmortem treatments, for example chilling methods, carcass suspension methods and Electrical Stimulation (ES) (Hope-Jones *et al*., 2010; Ellies-Oury *et al*., 2013; Marino *et al*., 2013; Kim *et al*., 2013; Hou *et al*., 2014; Chung and Davis, 2012). ES has been recommended as an effective way to improve beef tenderness and has been introduced to the beef industry as a standard slaughter process in many countries (Simmons *et al*., 2008). However, this technique has not been widely used in China. So, illumination of ES mechanisms will help to promote this technique in China which is the third largest beef producer in the world.

Previous research indicated that ES improved beef tenderness by three major ways. First, ES accelerated postmortem glycolysis, resulting in a rapid pH decline, which prevented cold shortening and consequently improved tenderness (Strydom *et al*., 2005; Hwang and Thompson, 2001). Second, ES activated the calpain proteolytic system and metabolic enzymes and thus enhanced the postmortem proteolysis of myofibril and cytoskeletal proteins (Strydom *et al*., 2005; Hwang and Thompson, 2001). Third, ES produced some contracted or stretched areas in myofibrils to damage the structure of the muscle tissue (Simmons *et al*., 2008; Luo *et al*., 2008). It can be seen from the above summary that the mechanism of ES improving tenderness is quite complicated; no single theory mentioned above can explain the mechanism comprehensively. However, the development of proteomics technology provides a new way to explore the mechanisms of beef tenderness with

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greater depth and insight (Bendixen *et al*., 2011; Bjarnadottir *et al*., 2011; Frylinck *et al*., 2009; Han and Wang, 2008; Hollung *et al*., 2007).

Our recent study investigated the protein changes in M. longissimus muscle at 3 days postmortem. Early postmortem is the most important time in the determination of meat quality (Marsh *et al*., 1981; Simmons *et al*., 2006). The purpose of this study was to identify differences in protein expression at 1 day postmortem as a result of ES, following procedures used in our recent study in order to more comprehensively understand the mechanism of ES tenderization and ultimately to improve beef tenderness of Chinese yellow crossbred bulls.

MATERIALS AND METHODS

Animals and sampling: Twenty Chinese yellow crossbred bulls (Yan-bian x Simmental, approximately 18 months old, live weight, 629.0±50.3 kg), fed under the same management from a commercial feedlot were transported to a commercial modern beef processing plant (Changchun Haoyue Islamic Meat Ltd. in Northeastern China). After fasting for 24 h with only water available, animals were randomly distributed into two treatment groups. Ten carcasses were Electrical Stimulated (ES) just after bleeding with an Electrical Stimulator (EST-601, Freund, Germany) for 40's (voltage, 42 V; electrical current, 0.7A; frequency, 50 Hz); the other 10 carcasses were Non-Stimulated as control (NS). Samples (10 g) for 2-DE analysis were removed from the M. longissimus at 24 h (between the 12 and 13th rib interface) postmortem by an expert butcher and stored at -80°C until assayed.

The experiment was undertaken following the guidelines of the animal ethics committee in Shandong Agricultural University and all experimental procedures were approved by the State Scientific and Technological Commission (China, 19881114).

Extraction of muscle proteins: Muscle protein extraction was done following the method by Kim *et al*. (2008). The 100 mg frozen muscle tissue was put in liquid nitrogen and pulverized. Then, the sample was put into 1 mL of protein extraction with protease inhibitor cocktail and incubated for 20 min. The protein extraction contained 8 M urea, 2 M thiourea, 65 mM dithiothreitol (DTT), 2% CHAPS, 1% IPG (immobilized pH gradient) buffer (pH3-10, GE). The samples were centrifuged at 10,000 g for 20 min and supernatants were used as the protein extract. To determine the protein concentration, a protein assay system (Bio-Rad, California, the United States) was used with Bovine Serum Albumin (BSA) as a standard.

Two-Dimensional gel Electrophoresis (2-DE): Two-dimensional gel electrophoresis was done following the method of Kim *et al*. (2008). Samples of approximately 400ig for analytical gels were loaded to 24 cm Immobilized PH Gradient (IPG) strips (pH 3-10, non-linear, GE). IPG strips were rehydrated overnight with a rehydration solution which contained 8 M urea, 0.5% CHAPS, 0.28% DTT, 1% IPG buffer (pH 3-10, GE), 10% glycerol and bromophenol blue. After rehydration, Isoelectric Focusing (IEF) was conducted for a total of 46,000 V-h using a PROTEAN IEF Cell unit (Bio-Rad). Voltage levels were stepped-up for 1 h, at 100, 200, 500 and 1000 V, respectively and then gradually increased to 8000 V. The current was limited to 50 mA/strip under 20°C. Total electrophoresis period was 6 h. In the following 20 min, IPG strips were incubated with 10 mL of equilibration solution, containing 50 mM Tris-HCl (pH 8.8), 6 M urea, 2% Sodium Dodecyl Sulfate (SDS), 20% glycerol, bromophenol blue and 5 mM TriButyl Phosphate (TBP). IPG strips were transferred to a 12.5% SDS polyacrylamide gel for electrophoresis and run at 10 mA per gel for 1 h, followed by 20 mA per gel. The electrophoresis continued until the dye front reached the bottom of the gel. Gels were fixed for 1 h in 40% methanol and 10% acetic acid, then they were visualized by Coomassie Brilliant Blue staining.

Stained gels were matched and analyzed using PDQuest Software (Bio-Rad). Each sample had three replicated gels and the gels normalized by the total quantity in valid spots and analyzed. The CBB-stained protein spots that demonstrated approximately two-fold or greater differences in staining densities between NS and ES treated groups were selected and the proteins were identified.

Mass Spectrometry (MS) analysis: The differentially expressed spots were excised from the CBB-stained gels and each protein was identified using the method described by Kim *et al*. (2008, 2013). In order to identify each protein, the protein molecular weights, isoelectric points and protein scores were considered.

RESULTS AND DISCUSSION

Approximate 650 spots were detected in the two-dimensional gels loaded with ES or NS muscle extracts (Fig. 1), using computer-assisted image analysis. A representative image of the 2-DE pattern is showed in Fig. 2. Six protein spots (identified by numbers) changed significantly in optical density in 1 day postmortem ES

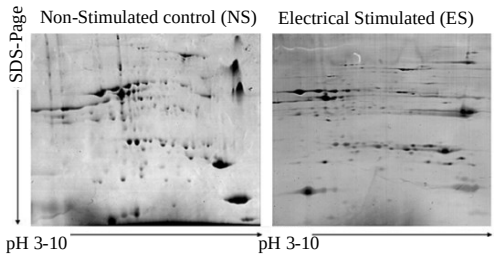

Fig. 1: Comparative analysis of the expressed protein patterns in Non-Stimulated (NS) and Electrically Stimulated (ES) M. longissimus of Chinese yellow crossbred bulls at 1 day postmortem. Protein (400 μg) was loaded and separated in the IPG pH 3-10 NL strip and an SDS gel (12.5% T)

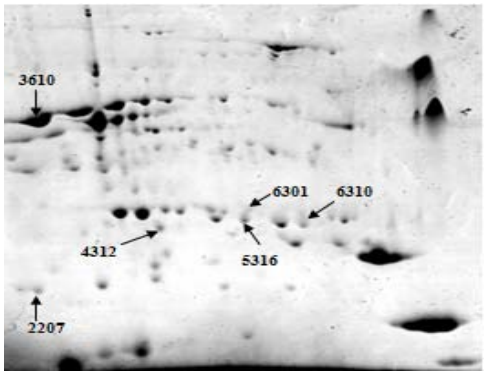

Fig. 2: Representative 2-DE pattern of the proteins extracted from M. longissimus of Chinese yellow crossbred bulls at 1 day postmortem. Six spots are numbered, respectively

samples, compared with the NS samples. The six points were identified based on their molecular weights, isoelectric points, molecular characterization and functions using the known protein databases (Table 1). They are desmin, troponin T alpha isoform, Phosphatidyl Ethanolamine-Binding Protein (PEBP), creatine kinase M chain and peroxiredoxin-6 (two spots). The optical density and hence the concentration of all six identified proteins was reduced significantly at 1 day postmortem as a result of the ES.

Our recent study identified another six proteins whose abundance decreased significantly at 3 days postmortem storage following beef carcass ES (Table 2). One unknown protein in that study was determined to be methyltransferase by our research group. The other proteins with lower concentration due to ES were myosin binding protein H, histone H3.3-like isoform 2, creatine kinase M-type and 2 spots identified as triosephosphateisomerase.

In this study and previous work from this lab, we observed a total of twelve protein spots (9 protein types) that were decreased significantly at 1 and 3 days postmortem as a result of ES. The ES protocol used in our recent study has also been found to significantly improve beef tenderness of Chinese yellow (Yan-bian x Simmental) crossbred bulls. ES tenderization of Chinese yellow bull muscle may be at least partially explained by lower concentration of proteins and enzymes of important metabolic pathways 1-4 (Fig. 3) identified by 2-DE as follows.

ES improved tenderness via the influence on the glycolytic metabolic pathway: In the present study,

Table 1: Identified differential proteins from M. longissimus of Chinese yellow crossbred bulls changed in abundance between NS and ES at 1 day postmortem

Spot No.	Protein name	NCBI accession No.	PMF (MS) score	Sequence coverage (%)	Theoretical molecular pI/mass (Da)
Cytoskeletal proteins					
3610	Desmin	gi|2959452	276	49	5.21/52530
6310	Troponin T alpha isoform	gi|1352725	195	35	9.53/27793
Kinase inhibitory protein					
2207	phosphatidylethanolamine-binding protein	gi|259913	277	73	6.96/20973
Metabolic enzymes					
4312	Creatine kinase M chain	gi|4838363	166	22	6.63/42944
Peroxidase					
5316	Peroxiredoxin-6	gi|5902790	231	50	6.00/25051
6301	Peroxiredoxin-6	gi|5902790	294	47	6.00/25051

Table 2: Identified differential proteins from M. longissimus of Chinese yellow crossbred bulls changed in abundance between NS and ES at 3 days postmortem

Spot No.	Protein name	NCBI accession No.	PMF (MS) score	Sequence coverage (%)	Theoretical molecular pI/mass (Da)
Cytoskeletal proteins, 2 of 7 proteins					
6703	Myosin binding protein H	gi|296479376	144	50	5.82/53686
6705	Histone H3.3-like isoform 2	gi|296202278	54	38	11.58/13636
Metabolic enzymes, 4 of 7 proteins					
3703	Creatine kinase M-type	gi|60097925	194	61	6.63/43190
4306	Triosephosphate isomerase	gi|61888856	210	82	6.45/26901
5310	Triosephosphate isomerase	gi|61888856	127	75	6.45/26901
Other					
3302	Methyltransferase	gi|116248579	80	35	6.23/24793

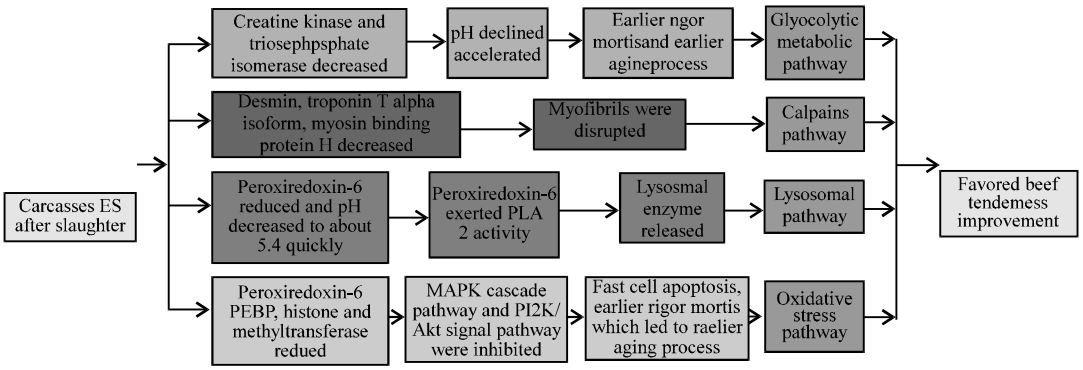

Fig. 3: ES improved beef tenderness by affecting four pathways identified by 2-DE

creatine kinase and triosephosphate isomerase showed lower abundance after ES. Consistent with the present result, Bjarnadottir *et al.* (2011) also found that the amount of creatine kinase in ES samples decreased. Muscle energy metabolism was transferred from aerobic to anaerobic metabolism after animals were slaughtered. ATP mainly comes from the processes that glycogen was degraded into lactic acid and that the phosphoric acid of creatine phosphate was transferred to ADP (Poso and Puolanne, 2005). In the glycolytic pathway, metabolic enzyme creatine kinase catalysed the reversible transfer of a phosphate group between ATP and ADP and triosephosphate isomerase catalysed the reversible transfer between glyceraldehyde 3-phosphate and dihydroxyacetone phosphate.

Creatine kinase and triosephosphate isomerase which showed lower abundance in ES samples indicated that these two metabolic enzymes were consumed rapidly during ES and larger amount of energy was produced through glycolytic pathway. And, it also reported that the fragment of creatine kinase increased in the insoluble protein fraction of Bovine Longissimus Thoracis muscle during the first 48 h postmortem (Bjarnadottir *et al.*, 2010). Thus which can be deduced here is ES could accelerate postmortem glycolysis resulting in a rapid pH decline and earlier development of rigor mortis in which metabolic enzymes have lower abundance (Dutson *et al.*, 1980). And the fast glycolysis indicates earlier aging process which in favor of beef tenderness (O'Halloran *et al.*, 1997).

ES improved tenderness via the influence on the calpains way: Tenderness depends on the architecture and the integrity of the skeletal muscle cell. In the present study, cytoskeletal proteins which include desmin, troponin-T and myosin binding protein H, showed lower expression abundance due to ES. It was also identified that desmin and troponin-T were decreased in ES samples (Bjarnadottir *et al.*, 2011). Troponin-t is an important part of skeletal muscle thin filaments and it takes part in regulating the thin filament during skeletal muscle contraction (Lehman *et al.*, 2001; McKay *et al.*, 1997). Desmin filaments surround the Z-lines of myofibrils and connect adjacent myofibrils at the level of their Z-lines and thus might played an important role in maintaining the structural integrity of muscle cells (Huff *et al.*, 2010). Myosin binding protein H can adjust the interaction between the myosin and actin (Bouley *et al.*, 2004). And these structural proteins are substrate of calpains (Goll *et al.*, 1983; Boehm *et al.*, 1998). The lower expression of desmin, troponin T alpha isoform and myosin binding protein H indicated that ES could activate the calpains pathway and accelerate the proteins degradation and finally improve beef tenderness. The result was consistent to our previous work that ES accelerated the rate of muscle protein degradation; desmin and troponin-T were degraded faster in ES samples analyzed by western blotting.

Furthermore, lots of previous work indicated that ES increased the calpains activity or reduced the calpastatin activity. This might can be explained by ES inducing post mortem glycolysis which caused a rapid pH decline and temperature increasing (Hope-Jones *et al.*, 2010; Dransfield, 1994). So, ES affected calpains system and increased the degradation of myofibrillar proteins such as desmin, troponin-T and then improve beef tenderness (Ho *et al.*, 1996, 1997; Rhee *et al.*, 2000).

ES improved tenderness via the influence on the lysosomal pathway: Peroxiredoxin-6 is an enzyme with glutathione peroxidase and phospholipase A2 (PLA2) activities which participates in antioxidant defense and hydrolyzes the sn-2 ester (alkyl) bond of phospholipids (Chen *et al.*, 2000; Fisher, 2011). Previous studies have reported that protein subcellular localization determined

the balance between the two activities of peroxiredoxin-6 on the basis of optimal pH. The PLA2 activity of peroxiredoxin-6 required an acidic pH at about 5.0 in lysosomes while the peroxidase activity required pH at around 7.0 in sarcoplasm (Manevich and Fisher, 2005; Wu *et al*., 2009).

In the present study, ES accelerated pH decrease to about 5.4. The decrease of peroxiredoxin-6 indicated that peroxiredoxin-6 exerted a Ca^{2+}-dependent PLA2 activity to breakdown the lysosomal membrane and release the hydrolytic enzymes to hydrolyze muscle proteins and improved tenderness (Fisher, 2011; Fisher and Dodia, 1996). ES and associated low pH stimulates calcium ion release from sarcoplasmic reticulum and mitochondria, which could stimulate lysosomal enzyme release (O'Halloran *et al*., 1997; Westerblad and Allen, 1991). Lower abundance of peroxiredoxin-6 could be an indication of higher degradation of this enzyme as its activity increases by ES and low pH. So, the effect of lysosomal enzyme on beef tenderness should be paid more attention under such circumstances that most researches focused on calpains system.

ES improved tenderness via the influence on oxidative stress pathway: Four proteins associated with oxidative stress pathways were identified in the present study, including peroxiredoxin-6, phosphatidyl ethanolamine-binding protein, histone and Methyltransferase and these four proteins take part in oxidative stress pathways mainly comprised by the MAPK cascade pathway and the PI3K/Akt signal pathway. The two pathways played critical roles in inhibiting oxidation and apoptosis(Kyriakis and Avruch, 2001; Kubo *et al*., 2008; Osaki *et al*., 2004; Song *et al*., 2005; Roux and Blenis, 2004). According to Schreurs *et al*. (1995), biochemical processes occurred in living tissue does not stop at the moment of death. The effect of ES on these proteins may increase cell apoptosis and accelerated rigor mortis and aging process and finally improved beef tenderness (Schreurs *et al*., 1995).

Peroxiredoxin-6 could play a peroxide oxidoreductase activity under neutral pH condition and in such circumstances, peroxiredoxin-6 could stimulate the proliferation and inhibit apoptosis through the p38 MAPK cascade pathway (Chen *et al*., 2000; Fisher, 2011; Manevich and Fisher, 2005; Kubo *et al*., 2008; Bast *et al*., 2010). In the present study, ES accelerated the decline of pH to about 5.4, so the glutathione peroxidase activity of peroxiredoxin-6 was limited. Furthermore, peroxiredoxin-6 was decreased as a result of ES. So, the effects of ES on peroxiredoxin-6 inhibited the p38 MAPK cascade pathway to some extent.

After slaughter, the Ras-MAPK cascade pathway was activated by the reactive oxygen species produced in the carcass (Valko *et al*., 2007; Hori and Nishida, 2009; Lee *et al*., 2009; Park *et al*., 2006). But PEBP can interact with Raf-1 to inhibited Ras-MAPK cascade pathway (Lee *et al*., 2009; Park *et al*., 2006). The decrease of PEBP by ES indicated that Ras-MAPK cascade pathway was inhibited. Histones recruit particular interacting proteins to complete the functions of apoptosis, nucleic acid metabolism, etc. In response to stress, histones can activate energy generation, accelerate protein synthesis and recruit anti-stress proteins to protect cells from degradation (Yang *et al*., 2010). Thus, histones may play a central role in stress response pathways involved in tenderization processes. The decrease of histones may indicate fast cell apoptosis and earlier rigor mortis. Histone methylation also plays an important role in the genomic imprinting, DNA repair and the gene transcription regulation (Bannister *et al*., 2001; Lachner *et al*., 2001; Tamaru and Selker, 2001; Smallwood *et al*., 2007; Zhao *et al*., 2009). And histone methylation needs Histone Methyl Transferase (HMT) which relates to the PI3K/Akt signal pathway (Cook *et al*., 2005; Greathouse *et al*., 2008; Bredfeldt *et al*., 2010). This pathway play a part of avoids apoptosis and activates MT (Bredfeldt *et al*., 2010; Torii *et al*., 2004; Crowell *et al*., 2007; Gonzalez *et al*., 2011). The lower the MT is expressed, the lower methylation is and the faster apoptosis is Zhang *et al*. (2002, 2012). Histone and MT were decreased by ES which indicated that the expression of MT and level of histone methylation was decreased and apoptosis was increased. Therefore, ES also inhibited PI3K/Akt signaling pathway to some extent.

Overall, ES accelerated oxidation and apoptosis by effect on MAPK cascade pathway and PI3K/Akt signal pathway and finally accelerated meat tenderization. The oxidative stress pathways of ES indicated that meat tenderness is closely related to oxidation and apoptosis. Those results tell us what should be foucsed on in further research thus, more experiments in our research group are ongoing to explore the effects of oxidative stress on beef tenderness and to verify the specific mechanism.

CONCLUSION

The present study identified 6 protein spots with lower expression decreased by ES at 1 day. Another 6 proteins were found to be decreased by ES at 3 days postmortem in a companion study from this laboratory. The proteins were of nine types: desmin, troponin T alpha isoform, myosin binding protein H, creatine kinase (2), triosephosphate isomerase (2); peroxiredoxin-6 (2), phosphatidyl ethanolamine-binding protein, histone H3.3-like 2 and methyltransferase.

Based on the protein function analysis, the improvements for the beef tenderness via ES were classified into four pathways; glycolysis, calpain release, lysosomal enzyme release and an oxidative stress pathway. Results suggest that ES accelerated pH decline and proteolysis, especially the degradation of cytoskeletal proteins. Furthermore, decrease of some oxidative enzymes suggests that an oxidative stress pathway is also an important way to affect beef tenderness. The results provided a more comprehensive understanding of the mechanism of how ES improved beef tenderness. However, the oxidative stress pathway is quite intricate and it is necessary to do further research to explore the specific mechanisms. Also, the release of lysosomal enzyme indicate that more attention should be paid on the contribution of lysosomal to beef tenderness and the oxidative stress pathway point out research direction and more experiments about the oxidative stress effect on meat tenderness has been designed and the specific mechanisms will be further verified.

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