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Full Length Article

Relationships between *DNAJA1* Expression and Beef Tenderness: Effects of Electrical Stimulation and *Post-mortem* Aging in two Muscles

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Abstract

Previous studies have demonstrated that *DNAJA1* could be a good indicator of beef toughness in *Longissimus thoracis* muscle from Charolais young bulls. This study aimed to check if relationship between *DNAJA1* expression and beef tenderness is valid in two muscles, which differ in toughness from Angus steers with or without electric stimulation at different times of *post-mortem* ageing. Electric stimulation did not significantly affect *DNAJA1* expression at the time of slaughter. But, RNA degradation soon after slaughter was a major factor, which affected the assessment of *DNAJA1* expression, even at 3 h *post-mortem*. Correlation of *DNAJA1* expression at slaughter with shear force values varied according to ageing time (24 h, 3, 7, 14 or 21 days *post-mortem*) or according to the use of electric stimulation and was not significantly correlated with any of these shear force peak values despite a few high correlation values (-0.60 or +0.34). In conclusion, *DNAJA1* is not an omnipotent marker of beef toughness with this particular data set since the correlation of its expression level with shear force values varies according to slaughtering conditions and ageing time. © 2015 Friends Science Publishers

Keywords: *DNAJA1*; Tenderness; Beef muscles; Electrical stimulation; Ageing

Introduction

Tenderness, juiciness and flavor affect beef palatability, with tenderness being the most economically important trait (Boleman *et al*., 1997). Due to advances in genomics, researchers have identified previously unrecognized genes associated with meat quality. Gene expression controls the biological characteristics of muscles. Knowledge of gene expression profiles has identified the gene *DNAJA1*, which is strongly and negatively related to tenderness, explaining up to 63% of variation in tenderness (Bernard *et al*., 2007). Study of gene expression in cattle may lead to improved understanding of *post-mortem* effects on beef quality (Eggen and Hocquette, 2004; Mullen *et al*., 2006).

The *DNAJA1* gene encodes a member of the 40 kDa heat shock protein family (Hsp40), which is a co-chaperone of the 70 kDa heat shock protein family (Hsp70) which affects protein folding and mitochondrial protein import. The *DNAJA1/Hsp70* complex inhibits ordered cellular death, or apoptosis (Bernard *et al*., 2007). After animal bleeding, cells enter anoxia and receive no more nutrients, thus initiating apoptosis. It has been hypothesized that apoptosis is the first stage of meat aging (Ouali *et al*., 2013), and a delay or inhibition of apoptosis by the *DNAJA1/Hsp 70* complex could reduce meat tenderization.

To achieve desirable organoleptic properties, meat is typically aged before consumption. *Post-mortem* tenderization of meat is a complicated process consisting of a series of biochemical pathways (Herrera-Mendez *et al*., 2006). However, high quality RNA is essential for gene expression studies and aging of meat may cause concern about RNA quality or integrity (Bojlul *et al*., 2007). Some studies have shown that a delay in tissue processing may not decrease RNA integrity as reflected by the abundance of messenger RNA (mRNA) (Marchuk *et al*., 1998; Seyboldt *et al*., 2003).

A common *post-mortem* treatment at large commercial beef abattoirs is electrical stimulation (ES) of carcasses, in which an electrical current passed through the carcass to accelerate anaerobic glycolysis and subsequent pH decline such that carcasses can be chilled more quickly without risk of cold-shortening (Bendall *et al*., 1976). Electrical stimulation has been shown to reduce Warner-Bratzler Shear Force (WBSF) values, improve tenderness, in beef, lamb and goat (Savell *et al*., 1977). As well, ES of beef carcasses has been shown to accelerate *post-mortem* tenderization (George *et al*., 1980).

The objective of this study was to relate *DNAJA1*

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expression to beef toughness in muscles that may differ in tenderness and to evaluate RNA integrity and expression of the *DNAJA1* gene during meat ageing in non-electrically and electrically stimulated muscles.

Materials and Methods

Animals and Harvesting Procedure

Five Angus steers were fed total mixed ration of corn silage, cracked corn, roasted soybeans, and feedlot vitamin and mineral mix for 258 days to a final average body weight 615±27 kg and approximately 15 months of age. Animals were slaughtered at the meat laboratory facility of the Department of Animal Science, College of Agriculture Sciences, Penn State University, PA, USA, in accordance of the USDA approved humane slaughter guidelines. After carcass dressing (ca. 20 min *post-mortem*), the right side of the carcass was electrically stimulated (ES, 350 V, 3 cycles, 30s per each cycle), the left side was non-stimulated (NES). Carcasses were weighed and average hot carcass weight was 369±15 kg, and then chilled directly at 4˚C for 24 h.

Muscles Samples and Storage

After 25 min *post-mortem* (zero time, 0 h) 300 mg muscle samples were collected from *Longissimus thoracis* (LT) and *Bicepes femoris* (BF) from ES and NES sides then homogenized in 3 mL TRIzol reagent using power homogenizer. Samples were directly kept at -80˚C until the next steps of RNA extraction. Further samples of the LD and BF muscles from ES and NES sides were taken at 1, 3, 6, 9 and 24 h *post-mortem*. After 24 h LD and BF muscles were dissected from each side and cut into steaks (2.5 cm thickness) that were vacuum packed, stored at 4˚C and sampled for RNA extraction following the same procedure at 3, 7, 14 and 21 d *post-mortem*. Steaks were kept frozen at -20˚C after completing their respective aging period for further analysis.

Extraction, Quantification and Purity of RNA

Total RNA was extracted from muscles samples (*n=200*) with TRIzol Reagent® (Molecular Research Centre, Inc. USA) according to the manufacturer's instructions. Re-extraction of RNA was carried out using chloroform-isopropyl alcohol. Extracted RNA pellet was washed once with 75% ethanol. The RNA pellet was finally dissolved in 50 µL of nuclease-free water. RNA sample concentration and purity were determined using a NanoDrop spectrophotometer. RNA concentrations were adjusted to be within the quantitative range of an Agilent RNA 6000 Nano Chip. Each RNA sample was analyzed on an Agilent Bioanalyzer with an RNA 6000 Nano Chip according to the manufacturer's instructions (Agilent Technologies, Santa Clara, CA).

Relative Level of *DNAJA1* Gene Expression

Primers and probes for *DNAJA1* gene (the genetic marker for beef tenderness) were designed by the Nucleic Acid Facility of the Pennsylvania State University using sequence data from Genbank and the real-time PCR probe/primer design software Primer Express (v2.0, Applied Biosystems). For real-time qPCR analysis performed by the Nucleic Acid Facility of the Pennsylvania State University, DNAse-treated RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City CA) and the protocol provided with the kit. Quantitation by real-time PCR was done by adding 10 or 20 ng of cDNA in a reaction with 2X TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City CA) in a volume of 20 µL. Primer was added at a concentration of 400 nM and a probe at 200 nM which was labeled with a 5' FAM and a 3' Black Hole Quencher (Biosearch Tech, Novato, CA). The amplification protocol consisted of 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and one min at 60°C in the 7300 Real-Time PCR System (Foster City CA). Ct values of the *DNAJA1* gene and the reference gene 18S were used with the delta Ct method (Livak and Schmittgen, 2001) to determine relative levels of *DNAJA1* gene expression.

Warner-Bratzler Shear Force (WSF)

The 1, 3, 7, 14 and 21 day steaks were evaluated for WBSF. Steaks were thawed at 4ºC for 24 h, packages were opened, and steaks were lightly blotted and then weighed. Steaks were wrapped in aluminum foil and cooked to 70ºC in a standard conventional oven set at 177ºC. Internal temperature at the approximate geometric center of each steak was monitored intermittently with a digital thermocouple thermometer (Electro-therm, Model HT 680A, Geraberg, Germany). Steaks were cooled to room temperature (24ºC) over a 4 h period. Three 1.3 cm diameter steak cores parallel to muscle fiber direction were removed from each steak by hand. Cores were sheared perpendicularly to muscle fiber direction using a Warner-Bratzler shear cell attached to a shear force instrument (Model TMS-90, Meat Shear Cell Model CW-2, Food Technology Corporation, Virginia, USA) with a crosshead speed of 250 mm/min. Peak shear force (kg force) and shear work (joules) were recorded then averaged by steak.

Statistical analyses

Comparisons between muscles with and without electric stimulation or between muscles from the same animals were achieved by the paired Student's *t* test. Pearson correlation coefficients between gene expression levels and shear force values were calculated for the combined data from both muscles using the statistical software (StatSoft Inc., Tulsa, OK, USA).

Results

Toughness of Beef

Shear force (either peak force or work) did not significantly differ between the two muscle types without electric stimulation. However, with electrical stimulation, a significant difference not only was observed in shear force (peak force and work) after 21 days of ageing, but also after 14 days of ageing for shear work values only (Table 1). Thus, for electrically stimulated beef, BF muscle was tougher than LT muscle at 14 and 21 days of ageing. The effect of electric stimulation was significant for peak shear force at 21 days of ageing for the LT muscle only (Table 1).

DNAJA1 Expression

DNAJA1 expression relative to that of 18S RNA did not significantly differ between muscle type and treatment (with or without electrical stimulation) at the time of slaughter. It is also highly variable except for *Longissimus thoracis* muscle without electrical stimulation (Table 2). Correlation between DNA expression values across muscles was 0.794 between the two treatments (with or without electrical stimulation) (Table 3). From 2 h onwards, total RNA from at least one sample was degraded and all of them were degraded at 21 days *post-mortem* (Fig. 1). *DNAJA1* expression was very low in samples with a high RNA degradation (data not shown). We therefore considered that the assessment of *DNAJA1* expression was not reliable except at the time of slaughter.

Correlations between *DNAJA1* Expression and Shear Force Value

On average, shear force peak values were poorly correlated across ageing times with or without electric stimulation (Table 3) except a few of them (PF24 h with PF14d, PF3d with PF7d, PF3d with PF3dES, PF7d with PF14dES). Correlations between shear force peak values and *DNAJA1* expression in the two muscles (n=10 values) varied according to time of ageing or to the use of electric stimulation (Table 3). Furthermore, *DNAJA1* expression values were not significantly correlated with shear force peak value at any time. However, because it was much higher than any other correlation, the correlation with PF14d ($r = -0.60$) with no electric stimulation is worth being mentioned even if it was not significant, as well the correlation of 0.34 between *DNAJA1* expression values with shear force values at 7 days of ageing after electric stimulation (Table 3).

Discussion

The biological mechanisms which determine meat tenderness have been studied for decades in order to find good biomarkers of tenderness which can be used as predictors in order to optimize livestock practices and slaughtering conditions with the ultimate goal to produce beef of the best palatability. Nevertheless, this research strategy was not productive despite some important discoveries: (i) meat ageing is a very important process for the ultimate beef tenderness, (ii) this process involves several proteolytic systems involved in the degradation of muscle proteins during ageing (calpain/calpastatin, cathepsin, proteasome and more recently described caspases and serpins) and (iii) more recently, it was discovered that the onset of apoptosis is among the first steps involved in the conversion of muscle into meat just after slaughtering (reviewed by Ouali *et al*., 2013). Therefore, looking for biological markers involved in the process of apoptosis became a recent active area of research. Furthermore, this approach became easier thanks to the development of genomic tools (transcriptomics, proteomics) through the examination of associated molecular signatures of all genes or proteins, which means that no working hypothesis regarding the underlying mechanisms is required (reviewed by Cassar-Malek *et al*., 2008).

So far, different research teams have identified markers of beef tenderness involved in the energy metabolism pathway, in cell detoxification, or which belong to the heat shock protein family or to the annexin family (reviewed by Ouali *et al*., 2013). Within the heat shock family, several markers were identified so far among, which we can cite the Hsp27 protein (Morzel *et al*., 2008) or the

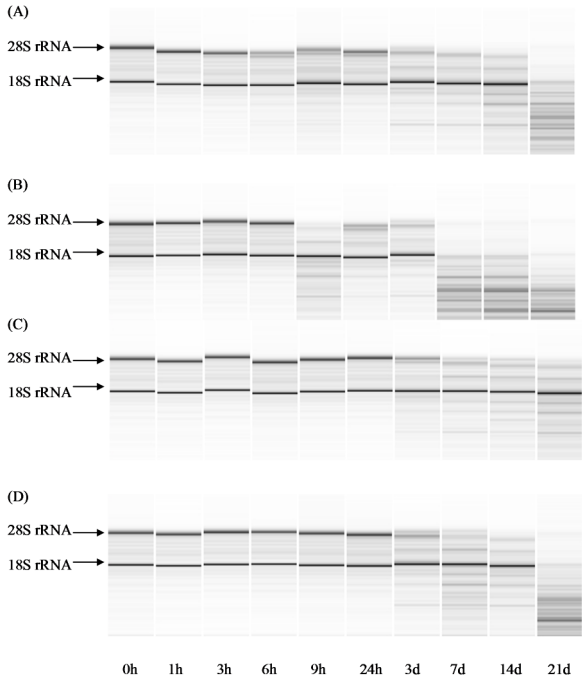

Fig. 1: Stability of total RNA in (A) Non-ES *Longissimus thoracis*, (B) ES *Longissimus thoracis,* (C) Non-ES *Biceps femoris* and (D) ES *Biceps femoris* muscles during *post-mortem* storage by Agilent Bio-analyzer assay

Table 1: Shear force[AA] of LT and BF muscles at 24 hours, 3, 7, 14 or 21 days of ageing

Variables		PF 24 h	SW 24 h	PF 3 d	SW 3 d	PF 7 d	SW 7 d	PF 14 d	SW 14 d	PF 21 d	SW 21 d
LT NES	Mean	43.10	46.30	41.90	44.70	37.10	41.90	38.30	43.80	32.90a	38.40
	SD	13.04	9.28	8.52	8.62	8.17	7.64	6.34	5.61	3.41	3.69
BF NES	Mean	37.90	46.70	41.90	48.40	48.40	50.10	38.30	48.70	32.90	41.90
	SD	14.55	17.82	12.32	17.30	9.90	17.68	6.98	9.52	6.59	8.99
LT ES	Mean	39.90	42.00	40.80	43.30	39.60	42.90	34.10	37.10A	28.6Ab	34.6 A
	SD	8.95	7.31	4.41	5.63	4.38	4.85	6.49	5.96	1.82	2.47
BF ES	Mean	39.40	46.80	40.80	46.80	35.40	46.80	39.50	43.00B	37.30B	45.8 B
	SD	4.10	4.58	6.02	8.03	4.22	7.43	3.71	4.87	3.62	5.20

[AA] Shear Force measured as Peak Force (PF, newtons) and Shear Work (SW, N cm)
A, B indicate differences of shear force values between LD and BF muscles for electrically stimulated muscles
a, b indicate differences of shear force values due to electrical stimulation for LT muscle
Means with different letters are significantly different (P<0.05)
LT: *Longissimus thoracis*, BF: *Biceps femoris*
NES: non-electrical stimulation. ES: electrical stimulation

Table 2: *DNAJA1* expression at the time of slaughter

Variables	Mean	SD	CV (%)
LT NES	1.02	0.01	1.40
BF NES	0.90	0.85	94.20
LT ES	0.54	0.43	79.40
BF ES	0.92	0.88	96.50

LT:*Longissimus thoracis*, BF: *Biceps femoris*
NES: non-electrical stimulation. ES: electrical stimulation
DNAJA1 expression levels are expressed in arbitrary units

DNAJA1 gene (Bernard *et al*., 2007). The DNAJ proteins (also known as heat shock protein 40) act as ascochaperones to the molecular chaperone DnaK (Hsp70), which is responsible for several cellular processes such as rescuing misfolded proteins, folding polypeptide chains, transport of polypeptides through membranes, assembly and disassembly of protein complexes and control of regulatory proteins (Qui *et al*., 2006; Jiang *et al*., 2007). In general, J-domain proteins modulate protein assembly, disassembly, and translocation (Walsh *et al*., 2004). DNAJ/Hsp40 proteins are indeed important for protein translation, folding, unfolding, translocation and degradation, primarily by stimulating the ATPase activity of chaperone proteins, Hsp70 (Qui *et al*., 2006). *DNAJA1* has been studied in biomedicine and has been shown to be a biomarker for pancreatic cancer (Stark *et al*., 2014).

In cattle, initial work has shown a strong negative relationship between *DNAJA1* expression in *Longissimus thoracis* muscle and tenderness score assessed by a sensory panel (Bernard *et al*., 2007). However, further work showed that *DNAJA1* was not the only gene belonging to the heat shock protein family related to tenderness and that the genes individually correlated with tenderness are not consistent across genders and slaughtering years in *Longissimus thoracis* muscle from the Charolais breed indicating the strong influence of rearing conditions on the relationships between *DNAJA1* expression and beef palatability (Hocquette *et al*., 2012). This may be explained by the fact that developmental age and management factors influence *DNAJA1* expression at the mRNA (Cassar-Malek *et al*., 2011) or protein levels (Guillemin *et al*., 2011). *DNAJA1* expression also differs between muscle types (Cassar-Malek *et al*., 2011). All together, the absence of consistency of relationship between *DNAJA1* expression with tenderness (across cattle population, age and muscle type) are likely to explain the absence of any association in the current study with Angus animals.

According to Fontanesi *et al*. (2011), transcriptomic studies can be performed in porcine skeletal muscle up to 24 h *post-mortem*. Therefore, we could conclude that microarray data obtained from specimens collected in the processing plant, over a relatively long period, have the potential to assess mRNA biomarkers of beef quality, with no potential bias from RNA degradation. In one way, our study confirmed this observation since most of the RNA degradation occurs after 1 or 3 days of ageing. However, careful examination of individual samples indicated that at least one sample RNA was degraded from two hours *post-mortem* onwards with a concomitant fall in *DNAJA1* expression. When performing correlation studies between two sets of data, any change in one point may affect the correlation dramatically, especially with a low number of samples. Therefore, the quality of RNA samples is surely a crucial factor determining the association between any potential biomarkers of tenderness and the real tenderness score.

Toughness (the opposite of tenderness) is routinely assessed by shear force on cooked meat. A first crucial parameter is cooking temperature which may be to an internal temperature of 55°C (rare cooking) as in France (Allais *et al*., 2011) or at 74°C in UK (Farag *et al*., 2008) or China (Hou *et al*., 2014). Shear force can also be measured after different days of ageing with poor correlations between shear force values depending on ageing time (Hou *et al*., 2014) as in this study (where correlations varies up to 0.69 maximum). Therefore, depending on the set of shear force values used to be correlated with potential biomarkers of beef quality, different results will be obtained. For instance, Tizioto *et al*. (2013) reported that *KCNJ11* SNPs were markers for tenderness using shear force values at 0 and 7 days *post-mortem* whereas *KCNJ11* expression level was a

Table 3: Correlations[A] between shear force[B] values and *DNAJA1* expression

Variables	NES DNAJA1	PF24h	PF3d	PF7d	PF14d	PF21d	ES DNAJA1	PF24hES	PF3dES	PF7dES	PF14dES	PF21dES
NES DNAJA1	1.00	-0.10	-0.05	-0.19	-0.60	-0.14	**0.79**	0.22	-0.27	0.15	-0.07	-0.08
PF24h	-0.10	1.00	0.16	-0.25	**0.69**	0.61	-0.22	0.11	0.55	-0.23	0.02	-0.39
PF3d	-0.05	0.16	1.00	**0.64**	0.17	0.46	0.16	0.30	**0.77**	0.04	0.46	-0.30
PF7d	-0.19	-0.25	**0.64**	1.00	0.17	-0.01	0.34	0.01	0.33	-0.18	**0.65**	0.39
PF14d	-0.60	**0.69**	0.17	0.17	1.00	0.46	-0.48	-0.16	0.43	-0.26	0.14	-0.13
PF21d	-0.14	0.61	0.46	-0.01	0.46	1.00	-0.21	0.04	**0.75**	-0.23	0.00	-0.30
ES DNAJA1	**0.79**	-0.22	0.16	0.34	-0.48	-0.21	1.00	0.05	-0.07	-0.08	0.21	0.24
PF24hES	0.22	0.11	0.30	0.01	-0.16	0.04	0.05	1.00	0.17	**0.69**	**0.66**	0.07
PF3dES	-0.27	0.55	**0.77**	0.33	0.43	**0.75**	-0.07	0.17	1.00	-0.14	0.25	-0.34
PF7dES	0.15	-0.23	0.04	-0.18	-0.26	-0.23	-0.08	**0.69**	-0.14	1.00	0.28	-0.12
PF14dES	-0.07	0.02	0.46	**0.65**	0.14	0.00	0.21	**0.66**	0.25	0.28	1.00	0.54
PF21dES	-0.08	-0.39	-0.30	0.39	-0.13	-0.30	0.24	0.07	-0.34	-0.12	0.54	1.00

[A]Correlations were calculated for the combined data from both muscles. Correlations in bold are significant at $P < 0.10$

[B]Warner-Bratzler Shear Force measured as Peak Force (PF)

NES: non-electrical stimulation. ES: electrical stimulation

negative marker of tenderness at 7 days of ageing only, but not at 0 or 14 days of ageing.

In our study, we found no significant relationships between *DNAJA1* expression levels and shear force values despite the highest negative correlation of -0.60 at 14 days of ageing. However, the major observation of this study is the high variability of this relationship between *DNAJA1* expression levels and shear force values which ranged from -0.60 to 0.34.

Many factors previously discussed are likely to explain at least in part this variability. Some technical factors related to the experiment itself are important such as any potential RNA degradation in at least one sample. The methodology used to generate reference values of toughness (in this case shear force) is also crucial since shear values vary with or without electric stimulation (this study), according to ageing time (this study, Tizioto *et al*., 2013; Hou *et al*., 2014) as well as according to other factors not studied here such as, for instance, suspension method of carcasses (Hou *et al*., 2014). In the case of ageing, biomarkers of tenderness (in this case protein expression levels because mRNA levels are not considered as reliable) vary according to ageing time (Laville *et al*., 2009).

Other authors argue that the reference values should not be shear force but sensory scores from expert panels. Indeed, phenotypic correlations between tenderness scores and shear force values were approximately -0.35 or -0.40 for French young bulls indicating that shear force is a poor indicator of the real tenderness assessed by real people eating beef (Van Wezemael *et al*., 2014). This problem, however, is not so important for genetic studies because the genetic correlations are much higher between these two parameters (around -0.90) (Allais *et al*., 2011). For both approaches, cooking method and cooking temperature in addition to muscle type affect shear force and tenderness values (Modzelewska-Kapituła *et al*., 2012). Generally speaking, consumers prefer the appearance, aroma and flavor of beef strip loin samples cooked at the highest temperatures and the tenderness and juiciness of samples cooked at the lowest temperatures (Gomes *et al*., 2014).

Other authors even think that the best descriptor of tenderness is the assessment by untrained consumers and not expert panels. Trained panels have generally a smaller variance due to a controlled methodology thanks to training, but the training procedure can lead to biased results. By comparison, a consumer panel is unbiased, but has a larger variance. The decision in Australia was to use untrained consumer taste panels and this decision was based on the need to have a reliable, transparent, system of testing samples that would engender confidence with both the beef industry and consumer sectors. In this country, the final assessment of palatability of beef is often done by the target consumer from the street eating beef (Thompson, 2002). Experiments relating biomarkers of beef quality to sensory scores determined by untrained consumers are still lacking, but results are likely to differ from those obtained so far with trained expert panels or with shear force as in this study. In contrast to previous results with one muscle type from young Charolais bulls, we observed that *DNAJA1* expression level was not a marker of beef tenderness with this particular data set made from two muscle types sampled from Angus steers. Besides animal differences (breed, gender, age, rearing practices), other technical factors related to the methodology (RNA degradation and storage), to the overall experimental design (muscle type, slaughtering conditions with or without electric stimulation) or to the assessment of tenderness (shear force *vs* score from expert panels) as well as temperature and cooking method of beef are likely to explain discrepancies between studies.

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