S3P02.WP

A SINGLE-LIMB INFUSION MODEL FOR INVESTIGATING POST-MORTEM TENDERIZATION IN BEEF

J.L. AALHUS[1], A. SZPACENKO[2], F.H. WOLFE[2], D.R. BEST[1]
and F. COSTELLO[1]

[1] Meat Research Section, Agriculture Canada, Bag Service 5000, Lacombe, Alberta, Canada
[2] Department of Food Science and Nutrition, University of Alberta, Edmonton, Alberta, Canada

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INTRODUCTION

Degradation of myofibrillar proteins in the post-mortem period is mainly responsible for the tenderization of meat which occurs post-mortem. However, the actual mechanism of protein degradation is still not clear and both the calpain and lysosomal enzyme systems, as well as non-enzymatic mechanisms have been implicated by various researchers (Koohmaraie, 1992; Zeece, 1992; Takahashi, 1992). Since the study of protein degradation under post-mortem conditions is confounded by between animal variation, the present research was directed towards the development of a within animal hind-limb infusion model to study protein degradation post-mortem in beef cattle.

MATERIALS AND METHODS

Infusion

The animal was stunned, shackled by both legs and exsanguinated. Immediately after bleeding the hide was cut and muscle samples (approximately 200g) were removed from the left *semimembranosus (SM)* and the left *biceps femoris* (BF) with a 34mm diameter stainless steel corer. The animal was then partially eviscerated (i.e., the abdominal cavity) and lowered to the floor. The femoral artery and vein in the right hind limb were isolated and the femoral vein was cut approximately 6cm proximal to the branch of the external iliac vein to limit return of the infusate to general circulation. The femoral artery was clamped approximately 10cm proximal to the external iliac artery branch and a small incision was made 3 to 4cm distal to the clamp to allow the insertion of a 75mm long, 4.5mm outside diameter, 3mm inside diameter, stainless steel needle. The infusion needle was secured in place and twenty litres of a treatment solution (37 °C) was infused into the right hind limb using a Baldor (Model FDL3504M) 0.5hp pump at approximately 15psi. After the infusion was complete the carcass was resuspended on the overhead rail and processed in the normal commercial manner. Muscle samples were collected from both the control (C) and treated (T) limbs 45 minutes, three and 10 hours post-mortem. The carcass was chilled overnight in a cooler (1 °C, wind speed 0.5m/sec) and after 24 hours the SM, BF and *semitendinosus* (ST) were dissected from the carcass and subsampled. The muscles were stored in plastic bags in a cooler until sampled at seven days post-mortem.

Measurements and assays

Temperature and pH were recorded for the C limb only at 0h and for both the C and T limbs at 45 minutes, three hours, 10 hours, 24 hours and seven days post-mortem at the sampling locations (BF and SM) using a puncture type electrode. Sarcomere lengths and diameters were measured at 24 hours and seven days on the C and T muscles (BF and SM). Muscle samples from the BF (0h-C only; three hours, 24 hours and seven days) were collected and split into

sarcoplasmic, sarcolemmal and myofibrillar fragments by differential centrifugation (Goll *et al.*, 1974). Total protein concentrations were determined on the protein fractions using the Pierce Chemicals Company BCA Protein Assay Reagent Kit (Rockford, Ill). An estimation of the amino group content was determined according to the ortho-phthaldialdehyde (OPA) method reported by Church *et al.* (1983) modified by replacement of 2-mercaptoethanol by N-acetyl-L-cysteine (Medina Hernández *et al.*, 1991). Slab sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on the protein fractions according to Laemmli (1970). As well, muscle samples from control and infused SM and BF were collected at 0 (C only), 45 minutes, three, 10 and 24 hours post-mortem, frozen with liquid nitrogen, and stored at -35°C until assays were performed. These samples were analyzed for glycogen, glucose, lactate and glucose-6-phosphate following the methods of Dalrymple and Hamm (1973) and Bergmeyer (1974) and for buffering capacity according to the method of McCutcheon *et al.* (1986) modified for use with larger samples. Drip loss was determined by weight difference on C and T steaks cut at 24 hours from the BF, SM and ST and held in a cooler (2°C) for six days. Warner-Bratzler shears were determined on cooked C and T steaks (internal temperature 72°C) from the BF, SM and ST at 24 hours and seven days post-mortem on an Instron Model 4301 Materials Testing System.

RESULTS AND DISCUSSION

Infusions with Evan's blue dye confirmed an excellent perfusion of the muscle tissue with the above described infusion technique, without incurring cross-over contamination between the C and T limb. Using the developed model, infusions of 0.9% saline (n=4; SA), water (at 2°C; n=2;WA), 0.3 M $CaCl_2$ (n=3; CA) and 0.01M EDTA (n=3; ED) were performed. With the exception of the SA infusion, all treatments resulted in severe muscle contractions during infusion. At the 45 minutes and three hours sampling times the CA infused limb was still noticeably contracted whereas in the ED infused limb the muscle was flaccid.

A summary of the differences between control and infused limbs are presented in Table 1. The WA, CA and ED treatments had many similar effects on post-mortem metabolism that did not occur in the SA treatment. Values for pH tended to be lower in the WA, CA and ED infused limbs following infusion and were significantly lower in the ED infused limbs at 45 minutes and three hours and in the CA infused limbs at three hours. Temperatures in the infused limbs tended to be slightly lower at 45 minutes, especially in the cold water infused limbs (-11.5%). As well, the glycolytic metabolites reflected an early acceleration of postmortem glycolysis (lower glycogen levels at 45 minutes and three hours and higher lactate levels at 45 minutes). These results suggest that infusion of non-isotonic solutions may have caused membrane depolarization, muscle contraction and an acceleration of postmortem glycolysis. Given the effect of rate of glycolysis and its associated pH decline on tenderness (Marsh *et al.*, 1987; Smulders *et al.*, 1990) it is tempting to hypothesize that the infusion of any non-isotonic solution will have a tenderizing effect. Indeed, in the WA infused limb, shear values at 24 hours were slightly lower than the control limb (-9.1%, P=0.16). However, in the CA infused limb shear values were 16.8% lower than in the control limb (P=0.03) at 24 hours and in the ED infused limb shear values were 18.4% higher than in the control limb (P=0.04) at seven days. The similarity in magnitude of these changes (-16.8% vs. +18.4%) while presumably increasing (CA) or decreasing (ED) the levels of calcium in the cell, provide convincing evidence that postmortem tenderization is controlled by calcium. Whether the increase in calcium by itself (Takahashi, 1992) or through the activation of the calpains (Koohmaraie, 1992) causes tenderization has not been resolved by the present work. Further studies utilizing enzyme blockers are in progress to address this question.

Since a considerable amount of research has attempted to relate sarcomere length to tenderness (e.g., Smulders *et al.*, 1990), the results for sarcomere length were noteworthy. At 24 hours, the CA infused limbs were more tender than the control limbs, yet had significantly shorter sarcomere lengths. After seven days aging, when the control limbs had tenderized to the same extent as the CA infused limbs, sarcomere lengths in the CA infused limbs were still significantly shorter. The ED treatment also showed a lack of relation between sarcomere length and shear force. Hence the calcium mediated tenderizing process appears to have no relation to sarcomere length.

Further examination by SDS-PAGE of the CA and ED treatments revealed differences in the banding patterns (Figure 1). For the sarcoplasmic proteins (Supernatant #1) there was an earlier disappearance of high molecular weight

polypeptide chains (MW ≈205kDa) in the treated compared to the control at three hours and 24 hours in both the CA and ED treatments (A, D). This decrease was accompanied by an increase in the amount of small polypeptides (≤10kDa) at the tracking dye interface at 24 hours in the CA treatment only. For the sarcolemmal proteins (Supernatant #5) there was an earlier disappearance of the 100kDa band (24 hours) and the 55kDa band (24 hours, seven days) in the control compared to the CA treated limb (B). In the ED treated limb these differences are not as apparent (E).

For the myofibrillar proteins, there does not appear to be a difference between control and infused limbs in the degradation rate of the very high molecular weight proteins, such as titin and nebulin, in either the CA or ED treatments at 24 hours (we would expect to see an earlier disappearance of this band in CA infused limbs given the 24-hour shear values). By seven days post-mortem the high molecular weight band is disappearing in both the control and CA infused limbs (C), which may be the breakdown in nebulin and titin associated with aging, but does not appear to correspond with tenderization (only the control limb tenderized between 24 hours and seven days). These results may indicate that the high molecular weight structural proteins are not involved in calcium induced tenderization. This finding is of interest since the present theories on the mechanism of calcium induced tenderization include postmortem degradation of titin and nebulin, either through the action of the calpains (Robson *et al.*, 1991) or through non-enzymatic calcium induced splitting of titin (connectin) into subfragments (Takahashi, 1992). Further studies utilizing lower density or gradient gels are required for better resolution of the high molecular weight proteins.

Also for the myofibrillar proteins, bands begin to appear earlier (three hours and 24 hours) in the 26-37kDa region in both the CA infused and ED infused limbs compared to their respective controls. In the CA treatment only, there appears to be a higher concentration of low molecular weight proteins at the tracking dye interface after 24 hours in the CA infused compared to the control, indicating a further breakdown of proteins. This observation is supported by data which indicates a higher proportion of polypeptides in the CA infused muscle at three and 24 hour (data not shown). As well, in the ED infused limbs, there is an earlier (24T vs 24C; F) disappearance of a band at 24kDa with a concomitant appearance of a band at 22kDa. By seven days, both the control and infused limbs have the 22kDa band.

CONCLUSION

The above described single-limb infusion model shows great promise as a means of studying the complex changes which occur during the post-mortem period which eventually result in tenderization of the meat. The model reduces inter-animal variation, decreasing the number of animals required, and provides enough sample to measure both meat quality traits (shear, drip loss) and a variety of indicators of protein degradation. Our preliminary results confirm calcium mediated tenderization postmortem. Further studies utilizing enzyme blockers in the infusate should distinguish whether it is the calcium ions alone, or calcium activation of the calpains which results in tenderization.

ACKNOWLEDGMENTS

Financial support for this project was provided by the Alberta Cattle Commission and the Alberta Agricultural Research Institute. The creative assistance of Mr. Don Brereton and Mr. Chuck Pimm in adapting the theoretical model to abattoir conditions is gratefully acknowledged.

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Table 1. Summary of differences between infused and control hind limbs.

	Cold Water (n=2) D P	0.9% NaCl (n=4) D P	0.3M CaCl2 (n=3) D P	0.01M EDTA (n=3) D P
pH				
45min	-2.2 0.25	1.2 0.11	-2.5 0.21	-9.4 <0.01
3h	-0.3 0.74	1.4 0.26	-6.1 0.05	-10.5 <0.01
10h	-1.2 0.46	0.5 0.78	-4.4 0.13	-1.4 0.56
24h	-1.6 0.11	-0.3 0.47	0.2 0.67	0.4 0.79
7d	0.5 0.38	0.0 1.00	1.3 0.01	1.9 0.20
Temp., °C				
45min	-11.5 0.12	-4.0 0.02	-3.6 0.20	-0.8 0.47
3h	-5.9 0.14	2.5 0.32	-2.7 0.45	2.9 0.65
10h	-11.6 0.37	-4.6 0.07	25.8 0.07	17.1 0.04
24h	11.1 0.36	0.0 1.00	9.1 0.31	6.9 0.38
Fibre, μ diameter				
24h	-1.4 0.86	7.1 0.10	1.8 0.68	-6.8 0.15
7d	-0.6 0.95	-5.1 0.11	5.2 0.14	-2.8 0.40
Sarcomere length,μm				
24h	4.9 0.50	2.2 0.23	-36.4 <0.01	-12.6 0.02
7d	0.3 0.98	-1.1 0.58	-24.1 0.04	6.0 0.35
Buffering capacity, $\mu M.ml^{-1}$				
45min	0.6 0.89	-7.4 0.07	3.3 0.44	-3.9 0.31
3h	-1.8 0.66	-10.1 0.01	-5.2 0.02	-10.4 <0.01
10h	-3.3 0.50	-10.2 0.02	-4.0 0.04	-1.6 0.75
24h	-2.4 0.66	-7.3 0.07	-5.9 0.14	-4.5 0.24
Glycogen, $mMole\ g^{-1}$				
45min	-20.1 0.02	-8.3 0.39	-21.0 0.01	-25.4 <0.01
3h	-14.5 0.24	-2.1 0.82	-27.3 0.02	-33.5 0.10
10h	35.4 0.12	50.9 0.02	-26.5 0.22	-11.9 0.66
24h	10.4 0.74	-1.2 0.95	25.4 0.08	3.4 0.71
Lactate, $mMole\ g^{-1}$				
45min	14.4 0.59	-28.7 0.10	37.5 0.06	35.5 0.22
3h	-13.7 0.41	-16.8 0.27	-4.4 0.80	4.7 0.86
10h	-38.3 0.12	-11.6 0.57	-3.3 0.82	4.1 0.85
24h	21.9 0.48	-6.7 0.44	-26.1 0.01	-16.0 0.25

Drip loss, mg.g-[1] 7d	18.4 0.58	-- --	55.3 0.09	211.1 <0.01
Shear, kg				
24h	-9.1 0.16	8.7 0.33	-16.8 0.03	-5.6 0.42
7d	-- --	-0.4 0.96	5.6 0.57	18.4 0.04

[1] Difference of control values from infused values as a percent of control values.