Effects of Postmortem Storage on the Ultrastructure of the Endomysium and Myofibrils in Normal and Callipyge Longissimus[1]

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ABSTRACT: These experiments were conducted to examine ultrastructural changes in longissimus from normal and callipyge lamb during 14 d of postmortem storage at 4°C. Six crossbred ewe lambs (½ Dorset × ½ Romanov) were grain-fed and slaughtered at approximately 250 d of age. Leg conformation score was the basis for classifying carcasses into normal and callipyge. The normal and callipyge longissimus had mean Warner-Bratzler shear force of 2.8 (2.7, 2.4, and 3.4) and 9.0 (12.2, 6.9, and 7.9) kg, respectively, after 14 d of postmortem storage. The results of transmission electron microscopy demonstrated ultrastructural changes, including sarcolemma detachment, loss of myofibril lateral attachments, and I-band breaks in normal longissimus. Detachment of sarcolemma from myofibrils occurred in both phenotypes, but it was delayed by several days in callipyge longissimus. Thus, the sarcolemma detachment seems not to contribute significantly to postmortem tenderization. The endomysium of both phenotypes did not change with postmortem storage. In normal longissimus, the percentage of fractured I-bands increased from 0% at d 1 to 11% at d 3 ($P < .05$) and did not change between 3 and 14 d (15%) postmortem ($P > .05$). However, postmortem storage did not affect (0 to 3%) the frequency of the I-band breaks in the callipyge longissimus ($P > .05$). Therefore, the break in the I-band region in postmortem muscle is a change that is associated with postmortem tenderization. We conclude that the major factor responsible for the toughness of meat from callipyge longissimus is the postmortem stability of myofibrils.

Key Words: Callipyge, Tenderness, Cytoskeleton, Myofibrils, Endomysium, Longissimus

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Introduction

Current evidence indicates that postmortem meat tenderization is primarily the result of calpain-mediated degradation of key myofibrillar proteins and the cytoskeleton (for reviews see Goll et al., 1983, 1992; Koohmaraie 1988, 1992a,b, 1995, 1996; Ouali, 1990, 1992). Most of the cytoskeletal proteins, such as desmin, vinculin, filamin, and dystrophin, as well as the sarcomere proteins titin and nebulin, are native substrates of μ- and m-calpain, and the degradation of vinculin and desmin has been shown to correlate with postmortem meat tenderization (Koohmaraie et al., 1995; Taylor et al., 1995). The result of the degradation of key myofibrillar proteins and the cytoskeleton is that myofiber linkages are broken and meat becomes tender.

A new animal model for testing the proposed mechanism of meat tenderization hypotheses is the callipyge lamb. Muscles from callipyge lamb do not tenderize as rapidly postmortem as muscles from normal lambs. Thus, callipyge meat is tough even after extended postmortem storage under refrigerated conditions (Koohmaraie et al., 1995, 1996, 1998; Field et al., 1996; Shackelford et al., 1997; Duckett et al., 1998). The major factor contributing to the toughness of callipyge longissimus seems to be a reduction in the activity of the calpain proteolytic system caused by an increase in calpastatin activity (Koohmaraie et al., 1995). As a result, calpain substrates including vinculin, desmin, and titin are degraded very slowly, and myofibers are not fractured (Koohmaraie et al., 1995). Field et al. (1996) concluded that high shear values of callipyge muscle are due to myofibrillar

[1]Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of other products that may also be suitable. The authors gratefully acknowledge the technical assistance of S. Hauver and B. Gardner and the secretarial assistance of M. Bierman.

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properties, not collagen content or collagen crosslinking.

The objective of this study was to determine ultrastructural differences between normal and callipyge muscle during postmortem storage and their relationship to differences in postmortem tenderization.

Materials and Methods

Animals

The Roman L. Hruska U.S. Meat Animal Research Center Animal Care and Use Committee approved the use of animals in this study. Dorset callipyge rams were mated to Romanov ewes not of the callipyge genotype. Crossbred (½ Dorset × ½ Romanov) ewe lambs (n = 6) were grain-fed and slaughtered at approximately 250 d of age. Carcasses were dressed conventionally, and hot carcass weight was measured (26.6 ± 1.8 kg). Based on leg conformation scores, three of the carcasses were determined to have the callipyge phenotype.

Warner-Bratzler Shear Force

For determination of Warner-Bratzler shear force, longissimus chops were vacuum-packaged, aged (4°C) until d 14 postmortem, frozen, and stored (−30°C) for up to 2 mo. Chops were thawed (4°C) and broiled on Farberware Open Hearth electrical broilers (Farberware, Bronx, NY) to an internal temperature of 70°C. Chops were chilled for 24 h at 4°C to facilitate removal of cores. Two cores (diameter = 1.27 cm) were removed from each chop parallel with the longitudinal orientation of the muscle fibers. Cores were sheared against parallel axis of fibers with a Warner-Bratzler attachment using an Instron Universal Testing Machine model 4411 (Instron, Canton, MA) with a 100-kg load cell and crosshead speed of 200 mm/min.

Muscle Ultrastructure

Samples for ultrastructural examinations were prepared as described previously (Taylor et al., 1995). Briefly, at 1, 3, 7, and 14 d postmortem, muscle samples that had been stored at 4°C were cut to approximately 2 × 3 × 10-mm cubes and fixed overnight by immersion in cold 2.5% glutaraldehyde in .1 *M* sodium cacodylate buffer, pH 7.3. Samples were stained *en bloc* with 1% tannic acid, postfixed in 1% osmium, *en bloc* stained with uranyl acetate in 25% ethanol, dehydrated in ethanol, and then embedded in Spurr's resin. For all samples, thin sections of the same thickness (based on a silver color in the knife boat) were cut parallel and perpendicular to the fibers, allowing examination of myofibers and endomysium. Sections were stained with uranyl acetate and lead citrate, and then examined using a Philips 420 transmission electron microscope (TEM).

Quantification of I-band breaks was done according to the procedure described by Ho et al. (1996). For each animal, two blocks were cut and stained. The I-band breaks were counted in five randomly chosen grid squares, and the first 100 I-bands encountered scored as intact or broken, for a total of 500 per animal. Quantification of myofiber attachment to sarcolemma was determined on the same sections by counting the number of sarcomeres that were attached to sarcolemma. Only regions with obvious Z and M bands were evaluated, and quantification was done at 19,500 magnifications for 500 randomly chosen sarcomeres per animal. An ANOVA was conducted for a 2 (phenotype) × 4 (time postmortem) factorially arranged completely randomized design. When the main effects of the interaction were significant, least squares means were separated using the PDIFF procedure (a pairwise *t*-test) of SAS (1988).

Results and Discussion

The purpose of the current study was to determine differences in ultrastructural changes in callipyge and normal lamb longissimus during postmortem storage at 4°C. Several recent studies have indicated that meat from callipyge lamb has a high shear force value and improves minimally during postmortem storage (Koohmaraie et al., 1995, 1998; Field et al., 1996; Shackelford et al., 1997; Duckett et al., 1998). The lack of response to postmortem storage is caused by minimal changes in the proteins whose degradation is responsible for postmortem tenderization (Koohmaraie et al., 1995). Neither collagen content nor collagen crosslinks are involved in the toughness of meat from callipyge lamb (Field et al., 1996). Thus, the hypothesis guiding this study was that postmortem storage causes limited changes in callipyge muscle tenderness because of less myofibril degradation. The reduced degradation of myofibrils is hypothesized to be caused by an elevated amount of calpastatin in affected muscles. The biochemical evidence supports this notion because calpain substrates, including vinculin, titin, desmin, nebulin, and troponin-T are degraded very slowly and to a lesser extent in callipyge (Koohmaraie et al., 1995).

The normal and callipyge longissimus of the lambs used for this experiment had mean Warner-Bratzler shear force of 2.8 (2.7, 2.4, and 3.4) and 9.0 (12.2, 6.9, and 7.9) kg, respectively, after 14 d of postmortem storage. We chose the longissimus for ultrastuctural examinations because its tenderness is most affected by callipyge phenotype (Shackelford et al., 1997).

The first major ultrastuctural change in longissimus of normal lamb is detachment of the sarcomere from the sarcolemma (Figure 1, Table 1). The interaction between the main effects (phenotype and

days postmortem) was not significant ($P > .05$). The proportion of the sarcomeres attached to the sarcolemma decreased with increasing duration of postmortem storage ($P < .05$). Even though 66% of the sarcomeres were attached to the sarcolemma at d 1, only 14% remained attached after 14 d of postmortem storage. Overall, a greater proportion of the sarcomeres were attached to sarcolemma in longissimus from callipyge than from normal lamb (47 vs 28; $P < .05$). Sarcomeres are linked to the sarcolemma by filamentous structures called *costameres* at the M-line and Z-disk level (for review see Price, 1991). The costameres are reported to contain numerous proteins, including vinculin, dystrophin, desmin, vimentin, clatherin, and ankyrin (for review see Price, 1991). Costameres have been shown to provide the mechanical linkage for transduction of forces generated by myofibrils to the extracellular matrix (Danowski et al., 1992). Thus, it is expected that degradation of costameres could play an important role in meat texture. Based on the results presented here, we cannot assign a significant role for sarcolemma detachment in meat tenderness. After 14 d of postmortem storage, 80% of the sarcomeres in callipyge

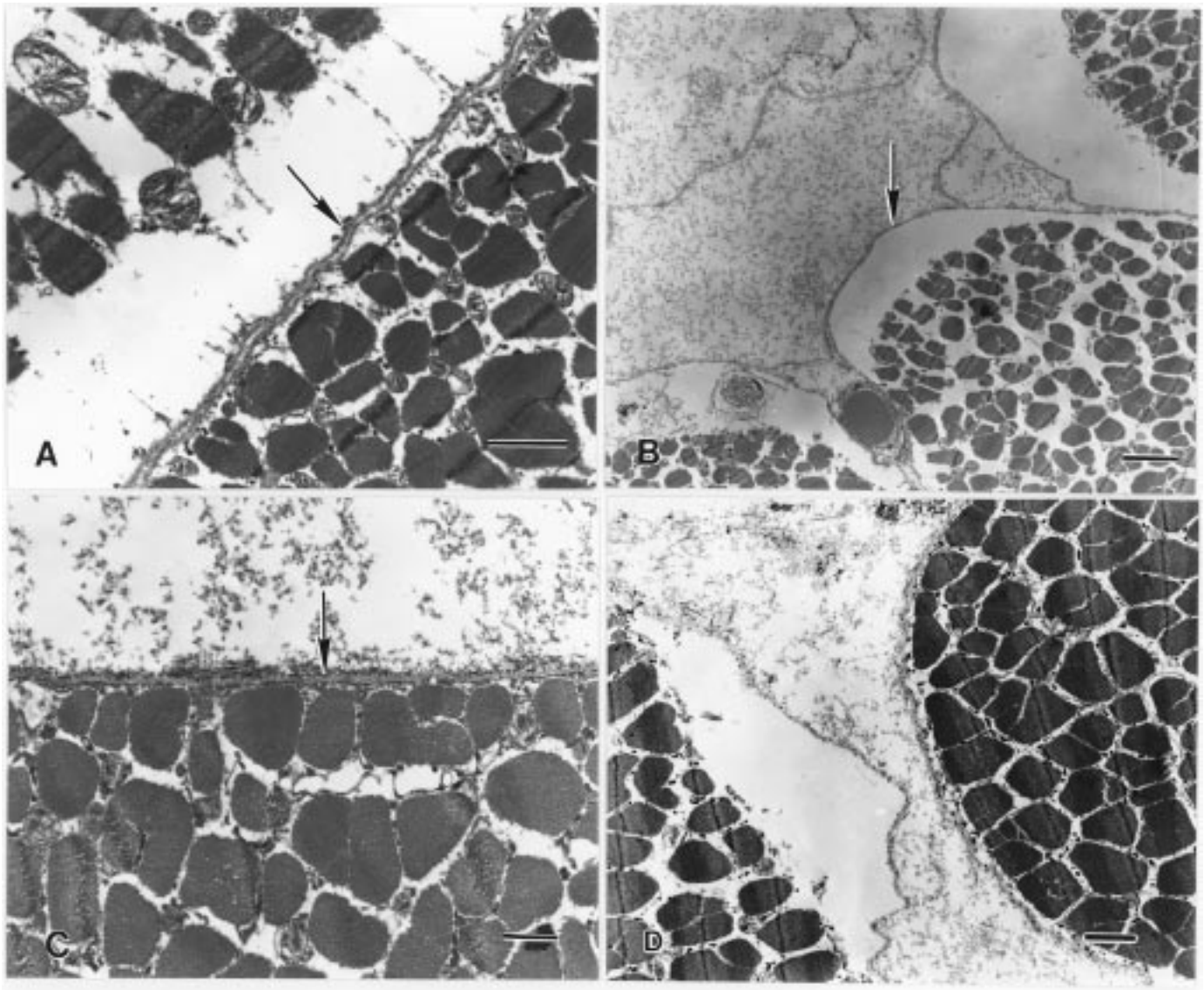

Figure 1. Electron micrographs of sarcolemma attachment to myofibers in lamb longissimus stored at 4°C. (A) Normal after 1 d of storage shows sarcolemma (arrows) attached to one fiber in the bottom half of the photo and detached from the upper fiber. The sarcolemma/endomysium from the two fibers remain attached to each other. (B) Normal after 7 d, showing that all of the sarcolemma (arrow) is detached. (C) Callipyge 1 d after death, showing that all of the sarcolemma (arrow) remains attached. (D) Callipyge after 7 d, with regions of attached (to fiber at right) and detached (fiber at bottom left) sarcolemma. Note that at all times shown there are no breaks in the basal lamina/endomysium. The bar represents 2 μm for A and B, 3 μm for C, and 1 μm for D.

Table 1. Percentage of sarcomeres attached to sarcolemma[a]

Phenotype	Phenotype means	Postmortem time, d 1	3	7	14
		66[x]	49[y]	22[z]	14[z]
		Individual means			
Normal	28[y]	52	42	11	7
Callipyge	47[x]	80	56	33	20

[a]Interaction not significant (P = .56); the SEM for interaction, main effect of days after death, and phenotype were 5, 8, 4.1, and 2.9, respectively.

[x,y,z]The main effects of phenotype and days after death were significant. Means lacking a common superscript letter differ (P < .05).

longissimus were not attached to the sarcolemma and yet the mean shear force value at the same time was 9 kg. The shear force of normal longissimus at 14 d postmortem was 2.7 kg and 93% of the sarcomeres were not attached to sarcolemma. The rate and extent of degradation of vinculin, a costameric protein, have been shown to be correlated with the rate and extent of postmortem tenderization (Koohmaraie et al., 1995; Taylor et al., 1995). However, the rate and extent of degradation of dystrophin, another costameric protein, does not seem to be correlated with the rate and extent of postmortem tenderization (Geesink and Koohmaraie, unpublished data). Until the precise supermolecular architecture of the costameric proteins is known, the difference in the rate and extent of degradation of vinculin and dystrophin and their relation to meat tenderness cannot be reconciled.

Another consistent change that occurs in muscle during postmortem tenderization is the fracture or breakage in the I-band adjacent to the Z-line (Davey and Dickson, 1970; Ouali, 1990; Taylor et al., 1995; Ho et al., 1996, 1997). These observations have suggested that this region of myofibrils is a susceptible site. We quantified the percentage of I-bands with breaks (Figure 2; Table 2). The interaction between the main effects (phenotype and days postmortem) was significant (P < .05). In callipyge longissimus, the percentages of fractured I-bands were 0, 1, 2, and 3 after 1, 3, 7, and 14 d of postmortem storage. By contrast, in the longissimus of normal lamb, the percentages of fractured I-bands were 0, 11, 13, and 15 after 1, 3, 7, and 14 d of postmortem storage (Table 1). The percentages of fractured I-bands increased from d 1 to d 3 (P < .05) and did not change between d 3 and d 14 postmortem (P > .05). However, postmortem storage did not affect the frequency of the I-band breaks in the callipyge longissimus (P > .05). These results are in agreement with those of Ho et al. (1996). Ho et al. (1996) reported that the frequency of the I-band breaks in beef longissimus was 1.8, 5.8, 6.5, 10.5, and 11.6% after 1, 3, 7, 14, and 28 d of postmortem storage. Because callipyge longissimus has a high shear force value that improves little with postmortem storage (Koohmaraie et al., 1995) and because the shear force value of normal longissimus decreases with postmortem storage, we conclude that the breakage in the I-band region of the myofibrils is a postmortem ultrastructural change that is associated with meat tenderization.

The last structure of interest was the endomysium. The endomysium is a thin collagen layer attached to the sarcolemma by the basal lamina (reviewed by Sanes, 1986). A number of studies have indicated that some changes occur in this structure during postmortem storage. Stanton and Light (1990) observed an increase in detergent-soluble endomysial collagen during postmortem storage. In the same study, some minor bands were observed in peptide maps of cyanogen bromide-digests of insoluble endomysial collagen of aged muscle that were not present when fresh muscle was used. This result indicates that some proteolysis of endomysial collagen occurs during postmortem storage. Using scanning electron microscopy and light microscopy, Liu et al. (1994, 1995) have shown that the endomysium of chicken muscle disintegrates postmortem and that this change seems to be associated with changes in tenderness. Results from the same group indicate that a weakening of the endomysium and perimysium of beef is a much slower process (Nishimura et al., 1998). A decrease in the mechanical strength of intramuscular connective tissue was observed only beyond 10 d of postmortem storage. Most of the tenderization of beef occurs before d 10. Lateral connections in endomysium contribute to translaminar shear (Purslow and Trotter, 1994), but these connections remain mechanically stable during postmortem storage (Willems and Purslow, 1997).

Light et al. (1985) reported that tough and tender muscles have the same amount of endomysium. Furthermore, endomysium does not seem to contribute to shear force value of single fibers (Mutungi et al., 1995). Therefore, it is doubtful that the endomysium plays a major role in determining the tenderness of meat. Because of the extreme toughness and reduced postmortem tenderization, callipyge longissimus represents an ideal model for determining the significance of changes in the endomysium. We hypothesized that endomysium, if related to shear force differences, should be more stable in callipyge.

The endomysium layer forms an integral unit with the basal lamina and the sarcolemma (Figure 3A), and detachment of the sarcolemma in longissimus of normal lamb resulted in no separation between any of these three layers (Figure 3B). However, the sarcolemma lipid bilayer was only partially preserved, even at postmortem d 1. The changes observed in the sarcolemma are probably due to artifacts of lipid extraction during sample preparation for electron

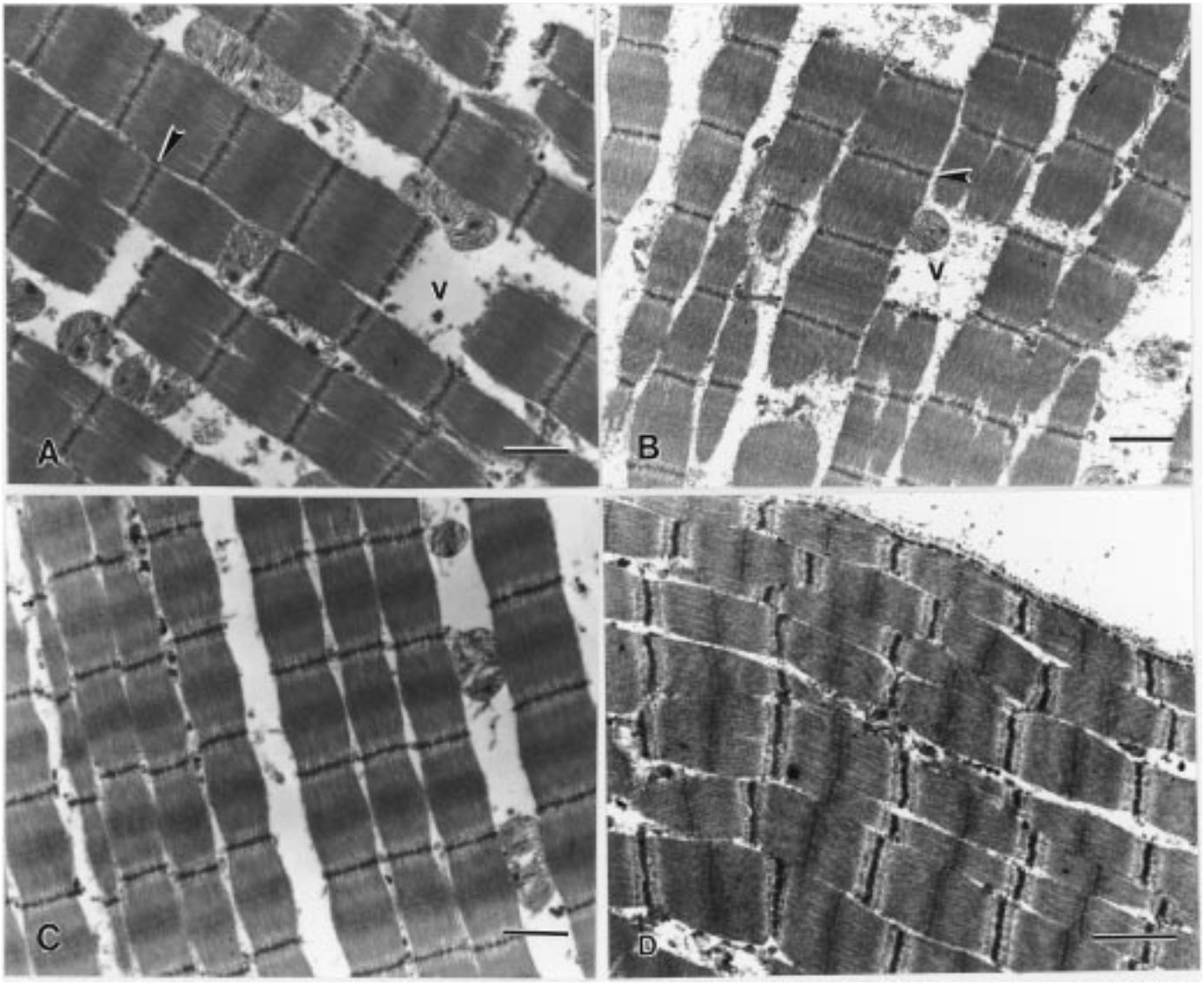

Figure 2. Electron micrograph of myofibrils at 14 d after death. (A) and (B) show postmortem changes in normal lamb longissimus with I-band breaks (∨), and loss of Z-line alignment (arrow). (C) and (D) show callipyge longissimus without I-band breaks. Bars represent 1 μm.

microscopy and postmortem changes. Therefore, most of what was visible consisted of the basal lamina attached to endomysium. At postmortem d 14, when normal longissimus has low-shear force value (i.e., tender), almost all of the sarcolemma/basal lamina/endomysium was detached (Table 1). Surprisingly, the basal lamina/endomysium was very stable at all postmortem times and consisted of an intact circle around the myofibers. We had expected that this thin layer of connective tissue would show some evidence of degradation, such as breaks and separation between the distinct layers, but this was not the case. As in normal longissimus, in callipyge longissimus the endomysium remained as an intact circle at all postmortem times (Figure 1b and Figure 3c). In normal and in callipyge longissimus, there were interconnections between the endomysium surrounding the individual fibers that remained intact at d 14 (Figure 3d). The results of this study indicate that the basal lamina/endomysium is stable for at least 14 d of postmortem storage in normal and in callipyge sheep.

Table 2. Frequency of I-band breaks[a]

	Postmortem time, d			
Phenotype	1	3	7	14
Normal	0^{y}	11^{x}	13^{x}	15^{x}
Callipyge	0^{y}	1^{y}	2^{y}	3^{y}

[a]Interaction significant ($P < .01$); the SEM for interaction was .8.
[x,y]Means lacking a common letter differ ($P < .05$).

The stability of endomysium in normal (low-shear force value, i.e., tender) and in callipyge (high-shear force value, i.e., tough longissimus) would suggest that this structure does not play an important role in postmortem tenderization. This interpretation agrees with the results of Light et al. (1985) and Mutungi et al. (1995).

Implications

The major ultrastructural difference related to the toughness of callipyge longissimus is the lack of change in muscle cytoskeleton and myofibrils. Changes in the endomysium were similar in callipyge and normal longissimus; thus, endomysium appears to

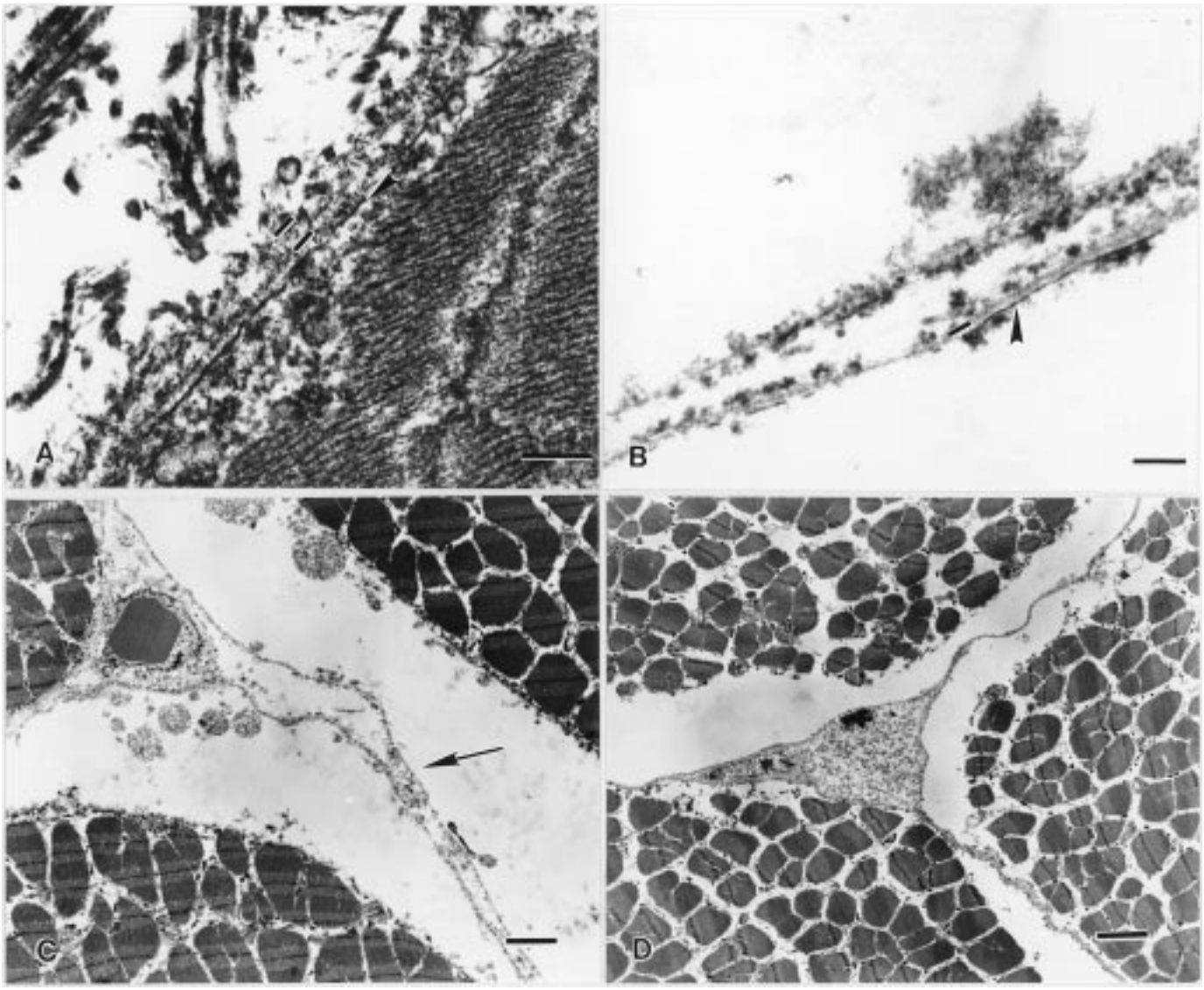

Figure 3. Electron micrographs of endomysium in postmortem longissimus. (A) high magnification of endomysium at 1 d. The sarcolemma (arrow) is attached to the basal lamina (—), which is attached to the endomysium layer (upper dash) containing collagen. Myofibers are in the lower right-hand corner, and fibrillar collagen is in the upper left part of the photo. In most regions, these three layers (referred to hereafter as the *endomysium*) were well-defined at 1 d for normal and for callipyge (shown). (B) After 14 d, the basal lamina and endomysium were still distinct. This micrograph shows two adjacent endomysia, which have remained attached. The arrow shows a portion of sarcolemma, and the dash indicates the basal lamina. The collagen layer is just above the dash. Note that the basal lamina is a continuous line and is intact. At 14 d, the structure of the basal lamina/endomysium was similar in normal (shown) and callipyge, both being stable. (C) Endomysium of 14-d callipyge longissimus. The micrograph shows that callipyge endomysium is intact and that often the adjacent endomysia are attached (arrow). Also note that three fibers (upper right, upper left, and bottom) are interconnected by continuous endomysium. (D) Normal longissimus at 7 d shows a region where three fibers (upper, lower, and right) are interconnected by endomysium. The endomysium is intact. Bar is .2 μm for A and B, 3 μm for C, and 4 μm for D.

have no role in meat tenderization. The rate and extent of sarcolemma detachment was reduced in callipyge relative to normal longissimus. However, sarcolemma detachment does not appear to significantly contribute to postmortem meat tenderization. These results support the hypothesis that postmortem tenderization is the result of the calpain-mediated proteolysis of proteins associated with the myofibrils and cytoskeleton. This implies that processes that activate calpain should improve or enhance, callipyge longissimus tenderness.

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