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Effects of Postmortem Freezing on Passive Properties of Rabbit Extensor Digtorum Longus Muscle Tendon Complex

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for Barbara A. Wilson, MAT, MS, DCA
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Frozen thawed EDL MTUs had significantly lower ultimate force to failure ($p<.01$), lower energy to failure ($p<.01$), lower strain at failure ($p<.0001$), and failed at a different anatomic location (fascia- muscle interface compared with the musculotendinous junction) compared with the EDL MTUs from freshly euthanized animals.

The results of this study have implications for the testing of freeze-stored muscles.

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The results of this study have implications for the testing of freeze-stored muscles.

EFFECTS OF POSTMORTEM FREEZING ON PASSIVE PROPERTIES OF RABBIT EXTENSOR DIGITORUM LONGUS MUSCLE TENDON COMPLEX

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Sometimes human and animal tissues must be stored until testing of biomechanical properties is feasible. This necessity has led to many studies investigating the changes which occur in tissue properties postmortem and after freeze-storage (1-10). Freezing is a common method of storing biological tissues (11,12). Woo et al. (13) have provided important data concerning the freeze storage of ligaments. They examined the structural properties of the rabbit medial collateral ligament (MCL)-bone complex and the mechanical properties of the MCL substance in freshly euthanized animals and in others that had been frozen from 1/2 months to 3 months at -20° C. After testing the femur-MCL-tibia specimens, they noted no statistically significant differences between fresh and stored samples in terms of load, deformation, and energy-absorbing capability at failure. Furthermore, they found that the stress-strain curves, tensile strength, and ultimate strain of the MCL substance did not change during freeze storage. They concluded that proper and careful storage by freezing has little effect on the biomechanical properties of ligaments.

To our knowledge, the biomechanical properties of musculotendinous units (MTUs) from freshly euthanized animals have not been compared with those of MTUs which have been stored in a frozen state. This study was devised to compare the strength properties of fresh and frozen MTUs and to determine if there is a difference.

Materials and Methods

Sixteen mature white male New Zealand rabbits with an average weight of 4.31 kg were used in this study. They were anesthetized with an intravenous mixture of

1cc of xylazine (20mg/cc) (Gemini, Rugby Laboratories, Rockville Laboratories, Rockville Centre, NY), 1cc of Ketamine (100mg/cc) (Vetalar, Parke-Davis Laboratories, Morris Plains, NJ) and 0.5cc of Acepromazine (10mg/cc) (PromAce, Ayerst Laboratories, New York, NY) titrated to physiologic anesthesia. Both hindlimbs were shaved and the right or the left hindlimb was randomly selected for the first operative procedure.

Under sterile conditions, using aseptic technique, the Tibialis Anterior muscle (TA) and the Extensor Digitorum Longus muscle (EDL) of the designated limb were exposed from their origins to their insertions into the foot. With the knee at 90° of flexion and the ankle (hock) at maximum dorsiflexion, a marking suture was placed around the EDL just proximal to the cruciate ligament (retinaculum) at the dorsum of the foot. The tendon of the TA was severed at its insertion at the base of the second metatarsal and the muscle was dissected to uncover the EDL. The multiple slips of the EDL tendon were severed distal to the metatarsal phalangeal joints. The EDL MTU was then dissected free to the level of its tendon of origin at the lateral femoral condyle of the distal femur. The EDL MTU was kept moist during this procedure by irrigation with normal saline.

The animal was then euthanized with 4cc of Pentobarbital (50mg/cc) (Nembutal, Abbott Laboratories, North Chicago, IL) and a 4.0 mm smooth Steinmann pin was passed through the distal femur at the level of the femoral condyles with a power drill. The animal was then transferred to the testing lab and the femur of the operated leg was fixed in a stereotaxis frame by securing the Steinmann pin with clamps. The distal tendons of the EDL were fixed in the grasping clamp of a United Testing Machine (United Calibration Corporation, Huntington Beach, CA) with the previously placed marking suture positioned at the lower end of the clamp. The EDL was vertically oriented with the Steinmann pin below and the grasping clamp above, and was pulled at an angle of approximately 135° to the long axis femur. (See Fig.1) The weight of the rabbit stabilized the femur.

Mechanical testing was initiated within 30 minutes from the time of death. The EDL MTU was continuously irrigated with a normal saline drip maintained at 38°C. Once the specimen was mounted, an initial mechanical rest length of the EDL MTU was determined. The crosshead of the mechanical testing machine was adjusted until the EDL MTU was visibly slack. The distance from the grasping clamp to the origin of the relaxed EDL on the femur was measured and recorded as the slack length. The EDL MTU was stretched 5 mm at 1000 mm/min and then returned to the slack length. All times, displacements, and forces were recorded on an IBM-compatible PC at.01 sec intervals. The distance required to generate a minimum positive force (15 grams) was read off the display monitor. This length was added to the slack length and recorded as the mechanical rest length (L_o). The crosshead of the mechanical testing apparatus was adjusted such that the distance from the origin of EDL to the grasping clamp equaled L_o.

After determining L_o, a series of "relaxation tests" were conducted. The EDL MTU was stretched from L_o to 104% of L_o at a velocity of 1000 mm/min, the EDL MTU was maintained at this stretch length for 7 minutes or until force reached steady-state. Data were collected at 10 samples/sec during this relaxation period. At the end of the relaxation period, the specimen was returned to L_o and allowed to rest for 2 minutes. This procedure was repeated for stretch lengths of 6%, 8%, and 10% of L_o. The EDL was then preconditioned by cycling it 10 times at 1000 mm/min between 110% of L_o and L_o. Immediately following the cyclic preconditioning, the EDL MTU was stretched to failure at a rate of 1000 mm/min. The force, time, and displacement at failure and the anatomic location of the failure were recorded. A typical force displacement curve is shown in Fig.2.

After mechanical testing, the rabbits were immediately eviscerated. The contralateral untested hindlimbs were splinted with the knee in extension (0° flexion) and the ankle at 90° of plantar flexion. The rabbit was then frozen at -80°C for 28 days in a sealed, air tight plastic bag.

After 28 days each rabbit, in its plastic bag, was thawed overnight in a 4°C refrigerator and then warmed prior to testing in a 38°C water bath for a minimum of one hour. Mechanical testing of the frozen thawed EDL MTU's was performed as described above for the fresh specimens.

Preliminary analysis of our data showed a shift in the apparent mechanical resting length (L_o) for a given specimen between tests. This shift indicated either that our mounting apparatus (stereotaxis) had shifted during testing or that we had not allowed enough time for the viscoelastic MTU to return to its original mechanical resting state. To investigate this problem, we conducted a series of tests substituting an electrical wire (22-gauge, insulated, and copper stranded) for a biological specimen. This wire behaved in a purely elastic fashion when mounted between a fixed lower clamp and the grasping clamp attached to the crosshead of the United Testing Machine. When mounted between the Steinmann pin (clamped in the stereotaxis apparatus) and the crosshead clamp, the wire behaved in a viscoelastic manner, generating characteristic stress-relaxation curves. Shifts in the apparent mechanical resting length between tests were also evident. From these observations, we concluded that the shifts observed in L_o, the apparent mechanical rest length, between tests for an individual specimen were the result of laxity in the Steinmann pin-stereotaxis mounting apparatus.

To correct for these shifts in the apparent mechanical resting length, we assumed that the calculated value of L_o was correct for the 4% relaxation test. Then we determined the displacement corresponding to 100 grams of force for each test. The correction in L_o between two tests (conducted at the same strain rate) was calculated from the difference between the displacement values corresponding to 100 grams of force. Corrected L_o values were incorporated into our calculations for energy to failure and strain at failure.

Statistics: The energy to failure, ultimate force at failure, and strain at failure for fresh and freeze

thawed specimens were statistically compared with a paired Students' t-test. The anatomical patterns at failure for fresh and freeze-thawed specimens were analyzed with Fisher's exact test.

Results

When testing the fresh specimens to failure, two EDL tendons slipped in the grasping clamp, one tore in the clamp, and two failed at the base of the clamp. Data from these specimens were not used in comparison of ultimate force at failure, strain at failure, energy absorbed at failure, and anatomical failure at break. In dissecting one frozen thawed specimen, a single slip of the EDL tendon was inadvertently severed, rendering that specimen untestable. In another frozen-thawed specimen, deep circular linear lytic changes were noted in the muscle belly of the EDL after thawing, rendering that specimen also untestable. One frozen-thawed specimen was palpated and noted to be excessively cold and was excluded from analysis because the distal tendon of its fresh contralateral counterpart tore in the grasping clamp with stretch to failure. Ultimate force and displacement data were not recorded in another fresh specimen. In eight additional specimens (fresh and frozen) insufficient data was captured to determine the energy absorbed to failure.

Data for five animals were available to compute energy absorbed to failure. Postmortem storage by freezing resulted in a significant reduction in the energy absorbed to failure ($p<.01$) with (mean$\pm$S.D.) 2.343 ± 0.471 Nm required for the fresh MTU's and 0.9183 ± 0.078 Nm required for the frozen MTU's.

Data from nine animals were available for analysis of ultimate force at failure and strain at failure. Postmortem storage by freezing resulted in a significant reduction ($p<.01$) in the ultimate force at failure from $17,047\pm2626$ grams for the fresh MTU's to $9,640\pm3428$ grams for the frozen MTU's. Postmortem storage by freezing was associated with a significant decrease in strain at failure ($p<.0001$) from

24.26%±4.20 for the fresh MTU's to 12.99%±2.24 for the frozen MTU's.

Ten animals were available to compare the anatomical location of failure at break. Eight of ten (80%) of the fresh specimens failed horizontally at the musculotendinous junction. Two of ten (20%) fresh specimens failed broadly at the interface between fascia and muscle. Ten (100%) of the frozen/thawed specimens failed broadly at the muscle fascia interface. (See Fig. 3) The difference between anatomical locations of failure is statistically significant at $p<.001$.

Discussion

The study of muscle injuries has lead to the investigation of the mechanical and structural properties of musculotendinous units. A previous study by Woo et al. indicated that the biomechanical properties of freeze stored ligaments are similar to those properties of ligaments from freshly euthanized animals (13). Our study indicates that the passive properties of frozen-thawed musculotendinous units are different from those of freshly euthanized animals.

Crisco et al. studied the strength of rabbit tibialis anterior and gastrocnemius muscles that had been stored at -20°C and then thawed to 37°C in a water bath. They found that the energy absorbed to failure and the stress failure increased more than 100% for a 10,000-fold increase in strain rate (14). Because our studies show that strength properties of the musculotendinous unit are significantly altered by the freeze storage process, we suggest caution in drawing conclusions from this study and others which test the mechanical properties of frozen-thawed muscle.

In our experiments, we placed a 4.0 mm Steinmann pin through the distal rabbit femur at the level of the femoral condyles. This pin was mounted in a modified stereotaxis apparatus. A similar type of testing apparatus has been used by other groups of investigators studying the properties of musculotendinous units (although some of these investigators were measuring forces of lesser magnitude) (15). Although convenient for mounting the rabbit femur, we found that this apparatus was not rigid and of itself added viscoelastic properties to the element being tested. The additional elastic components of the test system probably contributed to lower measurements of force than those that would be measured using a rigid mounting system at the same strain rate. The effects of the additional viscous elements are unknown.

All moveable or adjustable components of the stereotaxis apparatus were fixed as securely as possible. However, this was not sufficient to prevent

the apparatus from changing configuration slightly between tests, resulting in alterations in the apparent mechanical resting length. We corrected for these changes as described in the Materials and Methods section.

One frozen-thawed specimen, which was excluded from our analysis, was considerably colder to the touch than other frozen-thawed specimens. It was difficult to dissect, and was considerably stiffer with testing. For these reasons we recommend monitoring the temperature of the deep tissue throughout the thawing process.

Fresh specimens failed 80% of the time horizontally at the musculotendinous junction. These results are similar to those of Garret and McMaster(16,17). Frozen-thawed specimens always failed broadly at the muscle fascia interface. These results suggest that the freeze-thaw process alters the relative strengths of the muscle and tendon components of the EDL MTU. Further testing will be required to determine if the differences in the biomechanical properties between freshly euthanized and freeze/thawed MTUs is attributable to the muscular or tendinous portion of the specimen.

We conclude that the strength properties of musculotendinous units are altered by the freeze-thaw process. They become weaker (with a lower ultimate force to failure and lower energy absorbed to failure) and fail with a different anatomical pattern. We recommend that mounting systems for measuring large passive forces exerted on biological tissues be rigid and be tested, a priori, to determine if the mounting systems themselves exhibit elastic or viscoelastic behavior. Finally, we recommend that future experiments to determine the mechanical properties of muscle tissue be conducted either in vivo or using specimens from freshly euthanized animals.

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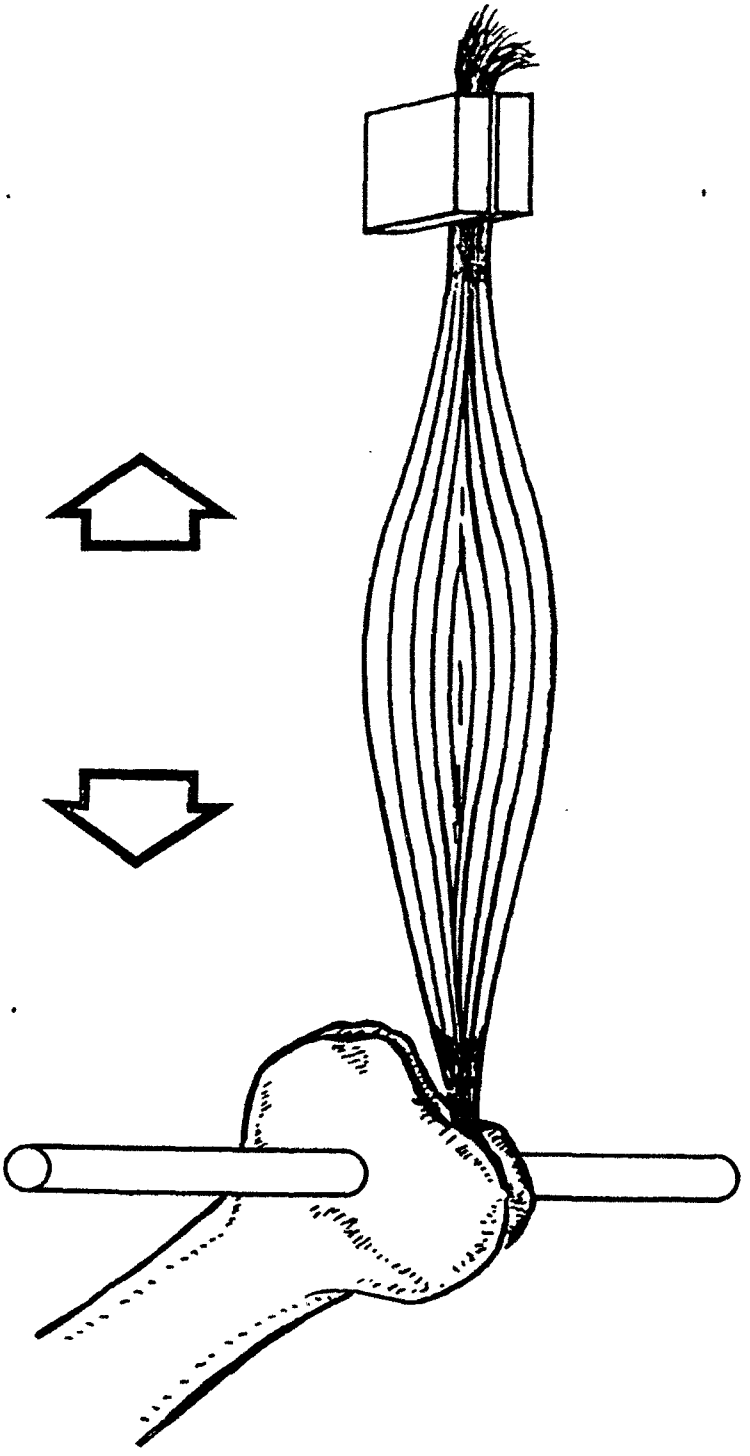

Fig.1 The EDL MTU mounted in the testing apparatus.

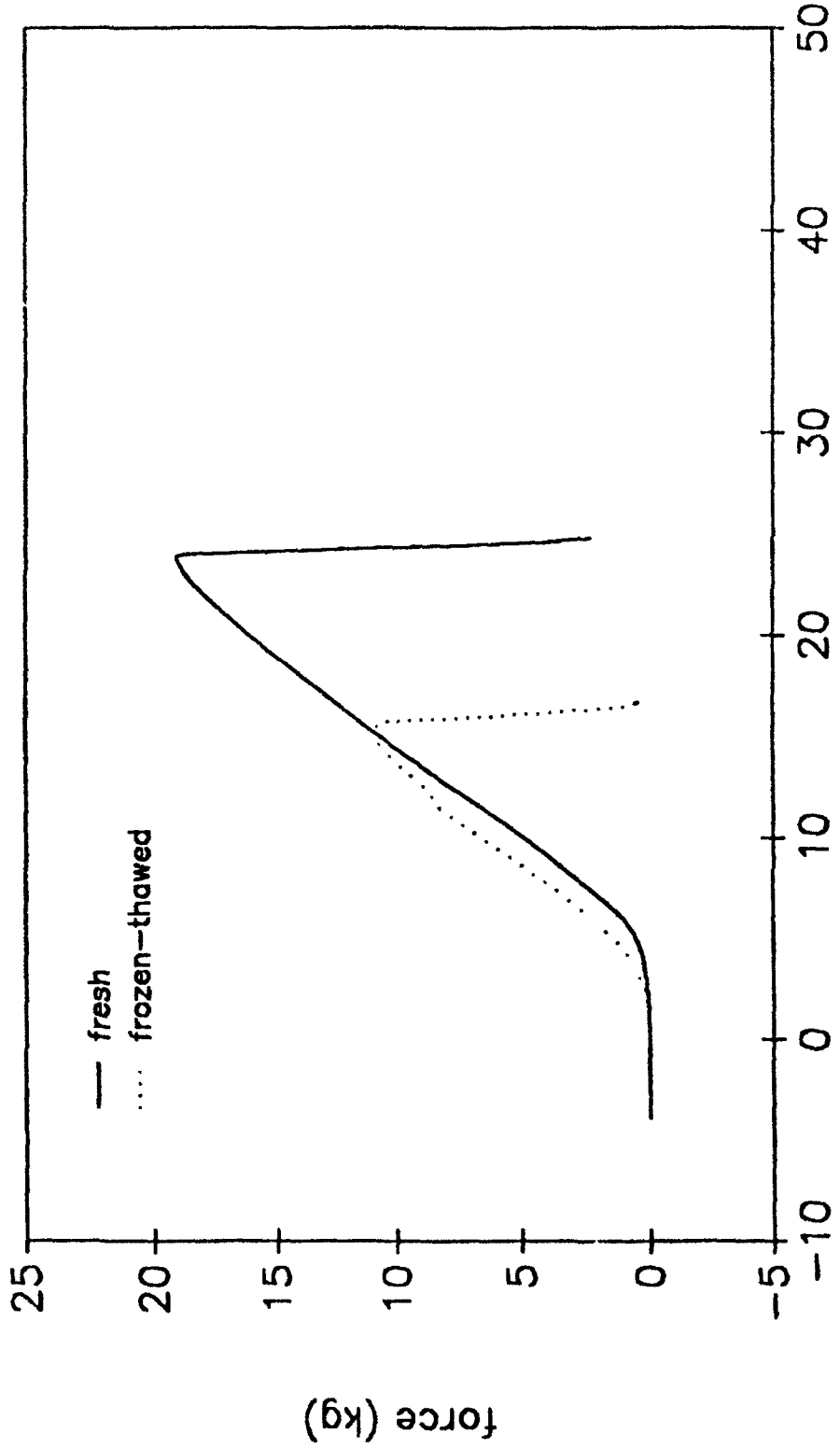

Fig.2 A typical force-displacement curve for a paired fresh and frozen-thawed EDL MTU.

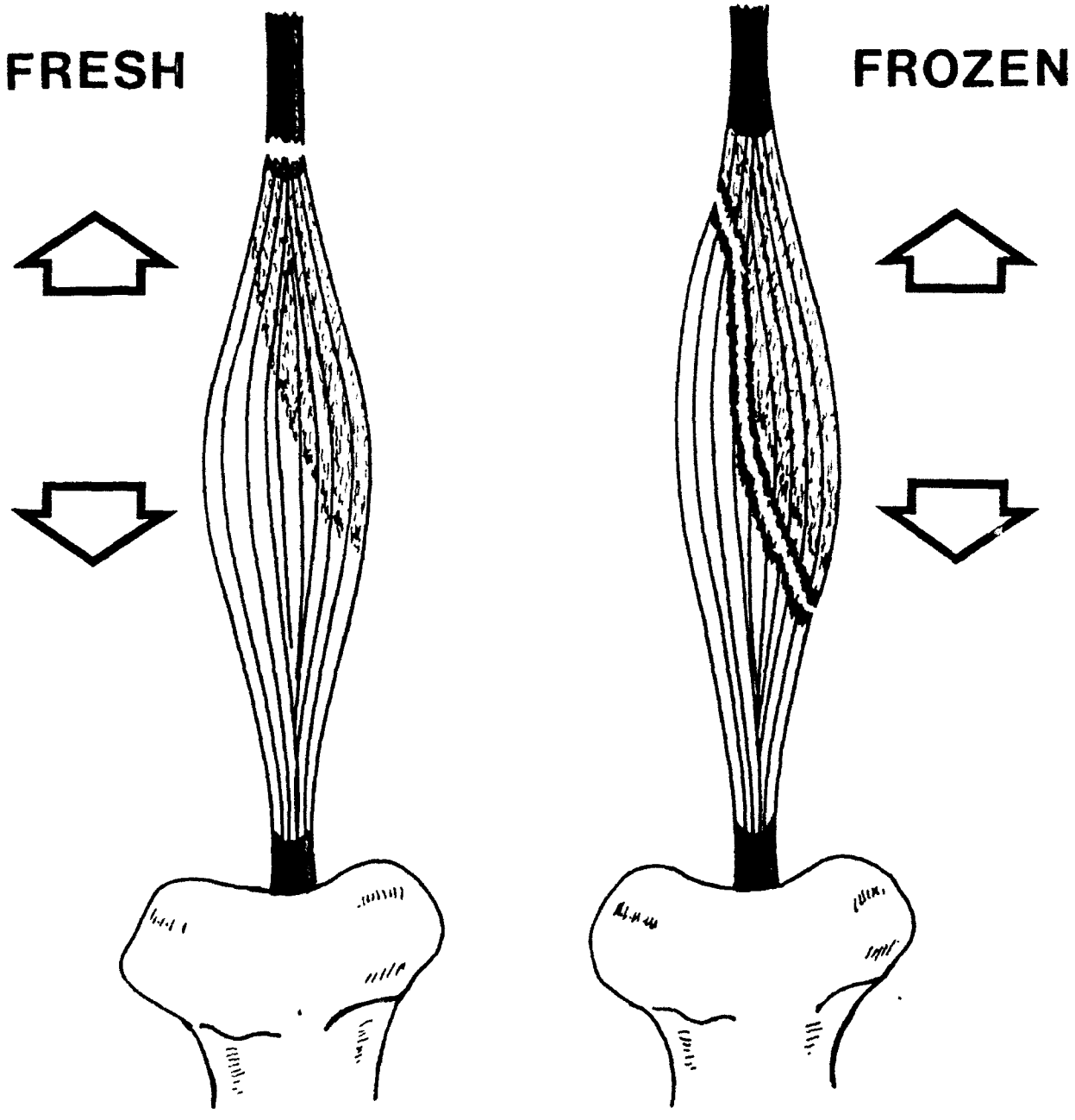

Fig.3 Tensile failure patterns for fresh and frozen-thawed EDL MTUs.

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