PREPARATION OF MUSCLE SAMPLES FOR ULTRASTRUCTURAL STUDIES AND CORRELATIVE MICROSCOPY (LM, SEM, TEM) OF SARCOMERE LENGTH AND SPACING OF INTERMYOFIBRILLAR CONNECTIONS.

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SUMMARY

Electron micrographs viewed in this study illustrate effects of seven different muscle sample preparations. Scanning Electron Microscopy (SEM) coupled with cold stage sublimation is an excellent technique for use in the study of meat and muscle ultrastructure. Moreover, these techniques can be used in correlative microscopy. Sarcomere length and spacing between intermyofibrillar connections were similar ($P < 0.05$) among electron microscopy preparation treatments and were changed similarly across heating treatments. Results indicate that the well sequenced transverse structures viewed by conventional SEM and the intermyofibrillar connections viewed by cryogenic SEM are probably Z-disks.

INTRODUCTION

During an investigation of heating rate effects on ultrastructural changes in prerigor muscle it was necessary to select methods of sample preparation which gave reasonably constant preservation and allowed us to make observations and measurements on structures whose identities were controversial (T-tubules, Z-disks, transverse structure, intermyofibrillar connections or bridges). Thus, SEM cold stage (SEM-CS) was used in correlation with SEM regular stage (SEM-RS) and transmission electron microscopy (TEM) to study the effects of seven different preparations on muscle preservation (qualitative correlation). Meanwhile, phase contrast microscopy (LM) was used with SEM-CS, SEM-RS and TEM to determine correlations among sarcomere length by (SA) LM and TEM (Z-disks), or SEM-RS (transverse structures) and spacing between intermyofibrillar connections by (SI) SEM-CS within constant areas of the same muscle sample (quantitative correlation) (Sybers and Ashraf 1973; Jones et al. 1976; Voyle 1981; Rowe 1984; Orcutt et al. 1986).

MATERIALS AND METHODS

Sampling and Treatment - Bovine *triceps brachii* muscle samples excised within 45 min of animal exsanguination, from 12 cattle (14-18 months age) and heated at different rates were used to evaluate sample preparations and intermicroscopic correlations (LM, SEM-CS, SEM-RS and TEM).

Sample Preparations - 1) SEM-CS Using Low and High Ice Sublimation - muscle samples (5 x 3 x 1.5 mm) were attached to aluminum stubs using carbon glue, plunged into nitrogen slush (-210°C), and stored overnight in liquid nitrogen. Samples were freeze-fractured in the cryogenic microscope prechamber, transferred to the main chamber of the SEM, then heated from -160°C to -82°C and held for 10 min (high ice sublimation) or to -88°C and held for 5 min (low ice sublimation). Samples were returned to the prechamber and coated with gold, observed and photographed using a JEOL-JSM-840 SEM-CS. 2) SEM-CS Using Fixative - samples described, were fixed in 2.5% glutaraldehyde, washed, attached to aluminum stubs, frozen in nitrogen slush (-210°C) and prepared as described previously, using high ice sublimation. 3) SEM-CS Using Cryoprotectant - samples were placed in cryogenic liquid (25% glycerol) for 15 h at 4°C, washed, attached to aluminum stubs, frozen in nitrogen slush (-210°C) and treated as described previously, using low ice sublimation. 4) SEM-RS Using Freeze-Substitution-samples were frozen in nitrogen slush (-210°C), using procedures modified from Orcutt et al. (1986). 5) SEM-RS Using Fixatives and Freeze-Fracturing - samples were fixed conventionally following procedures modified from Jones et al. (1976). 6) Transmission Electron Microscopy (TEM) - thin samples were primary-fixed in 2.5% glutaraldehyde, washed, secondary-fixed (2% $0s0_4$), washed and prestained. They were dehydrated using ethanol and acetone, embedded in Spurr's modified media, thin sectioned (60-90 nm), stained, washed, observed and photographed using a PHILLPS EM-200 TEM. Correlative microscopy (LM, SEM-CS, SEM-RS, TEM) of sarcomere length and the spacing between intermyofibrillar connections was carried out as described by Silva (1988).

RESULTS AND DISCUSSION

Effects of SEM-CS on Muscle Ultrastructure Preservation - Micrographs 1a and 1b represent high ice sublimation and show open spaces between myofibrils in longitudinal sections and several pores of ice damage in cross sections. Micrograph 1a shows well preserved sarcolemmae, myofibrils and allows us to make quantitative measurement of SI. Micrographs 2a and 2b represent low ice sublimation. Interpretation or quantitative measurements were not possible due to poor resolution of muscle structures. Micrographs 3a and 3b represent muscle that was fixed with 2.5% glutaraldehyde before freezing. Poor preservation of myofibrils and transverse structures and charging was observed in all sections. Micrographs 4a and 4b represent muscle that was treated with cryoprotectant (25% glycerol) before freezing. They show good preservation of muscle cells, sarcolemmae and connective tissues.

Effects of SEM-RS and TEM on Muscle Ultrastructure Preservation - Micrographs 5a and 5b represent muscle that was prepared by freeze-substitution-SEM-RS. Micrograph 5a shows a well preserved pattern of IC and smaller filaments (F). However, myofibrillar shrinkage was evident as described previously by Orcutt et al. (1986). Moreover, the spacing of IC (SI) was shorter (about 38.5%) compared to CS-high ice sublimation. Micrographs 6a and 6b represent muscle that was fixed/freeze-fractured-SEM-RS. Micrograph 6a shows excellent preservation of myofibrils, transverse structures (or Z-disks), collagen fibrils and sarcolemmae. This method permits interpretation and quantitative measurements of SA. Micrograph 6b also shows adequate preservation of muscle cells and collagenous

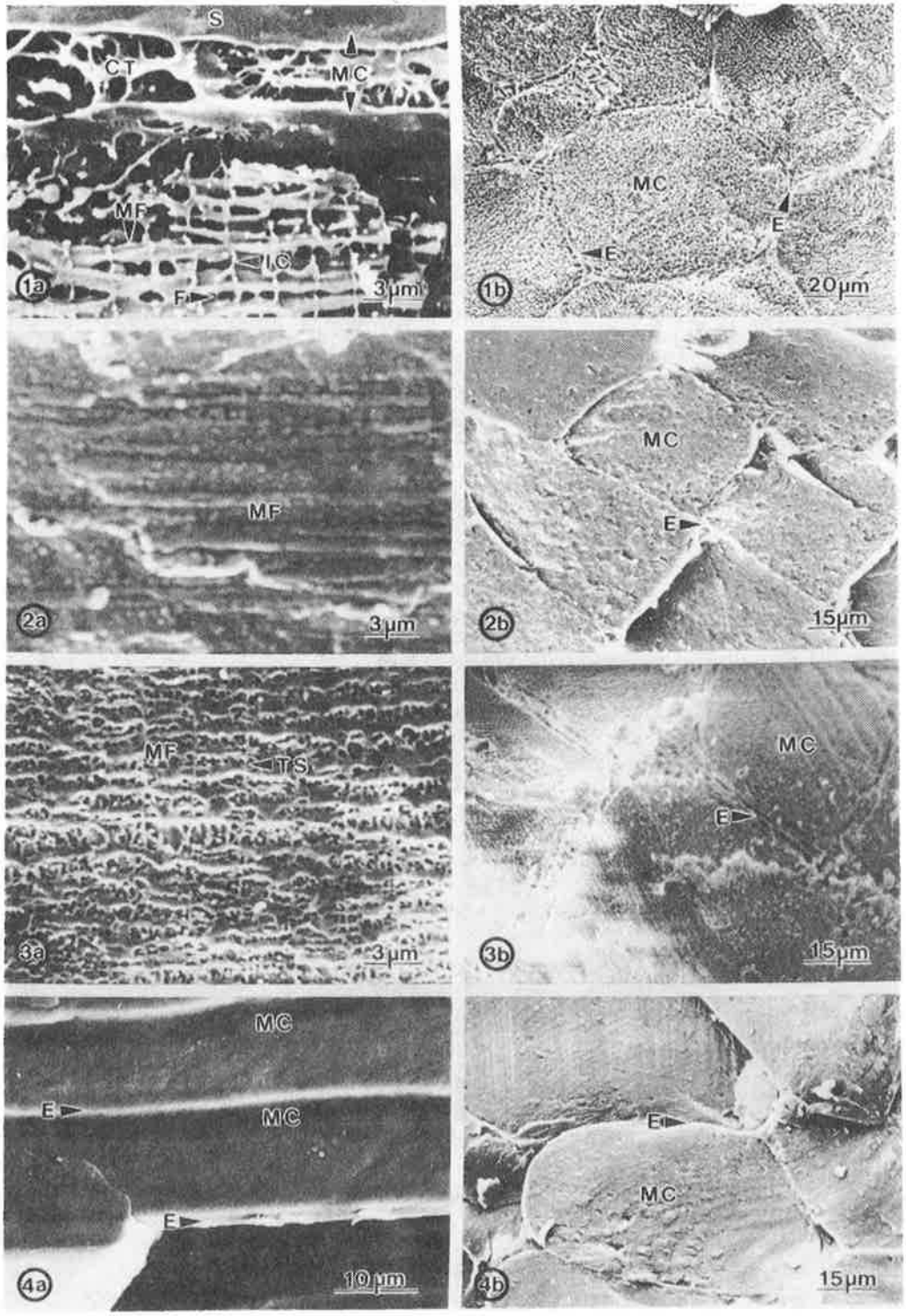

Figure 1. Cold stage scanning electron microscope micrographs of pre-rigor beef triceps brachii muscle: (1a, 1b) high ice sublimation; (2a, 2b) low ice sublimation; (3a, 3b) 2.5% glutaraldehyde as fixative; (4a, 4b) 25% glycerol as cryoprotectant. Sarcolemma (S), muscle cell (MC), connective tissue (CT), myofibril (MF), intermyofibrillar connections (IC), transverse structures (TS), small filament (F) and endomysium (E). a = longitudinal; b = cross section.

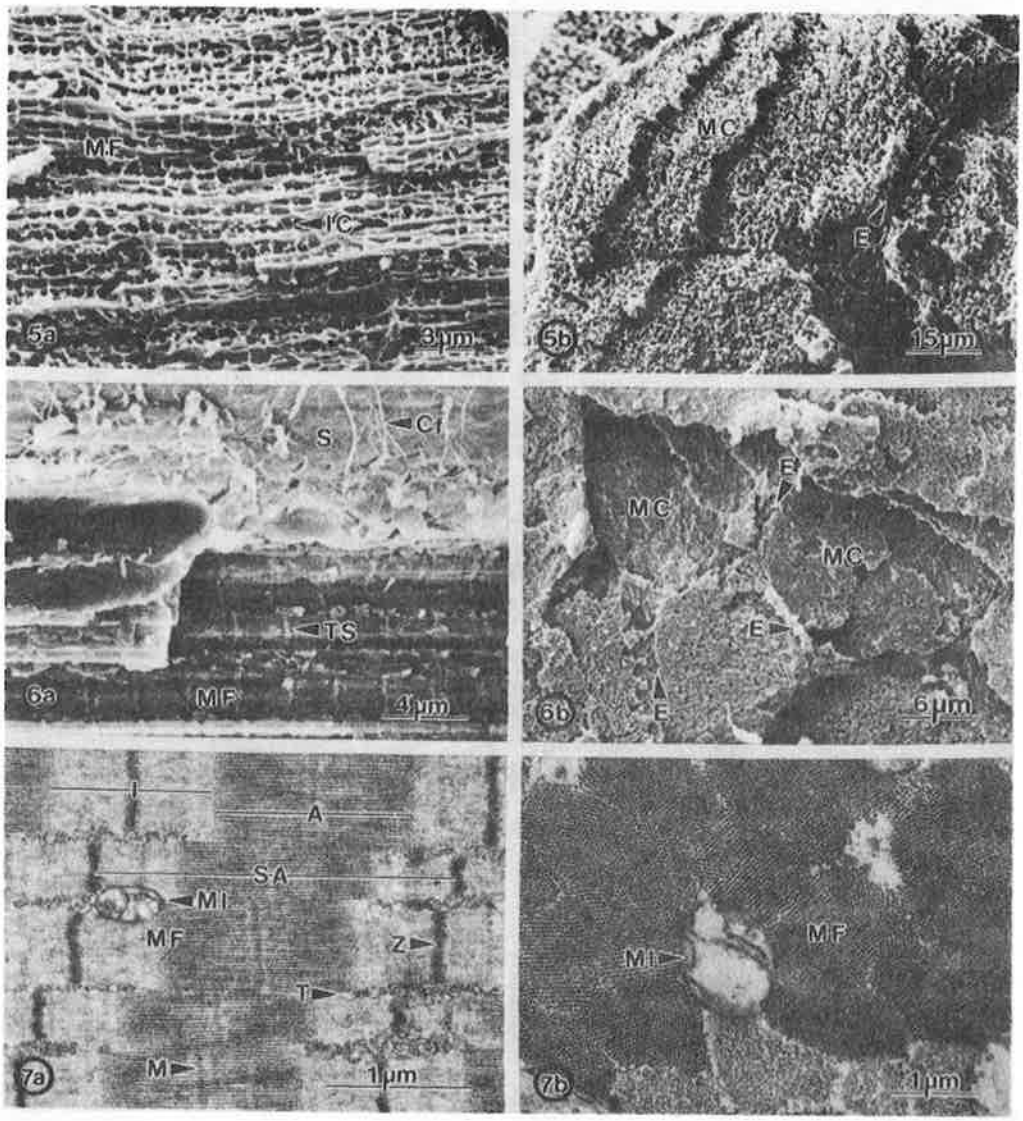

Figure 2. Regular stage scanning and transmission electron micrographs of pre-rigor beef triceps brachii muscle. (5a, 5b) freeze-substitution and scanning electron microscopy; (6a, 6b) fixation, freeze fracturing and scanning electron microscopy; (7a, 7b) regular transmission electron microscopy. Myofibril (MF), intermyofibrillar connections (IC), muscle cell (MC), endomysium (E), sarcolemma (S), collagen fibrils (Cf), transverse structure (TS), Z-disk (Z), I-band (I), A-band (A), M-line (M), triad (T), mitochondria (MI), sarcomere (SA). A = longitudinal; b = cross section.

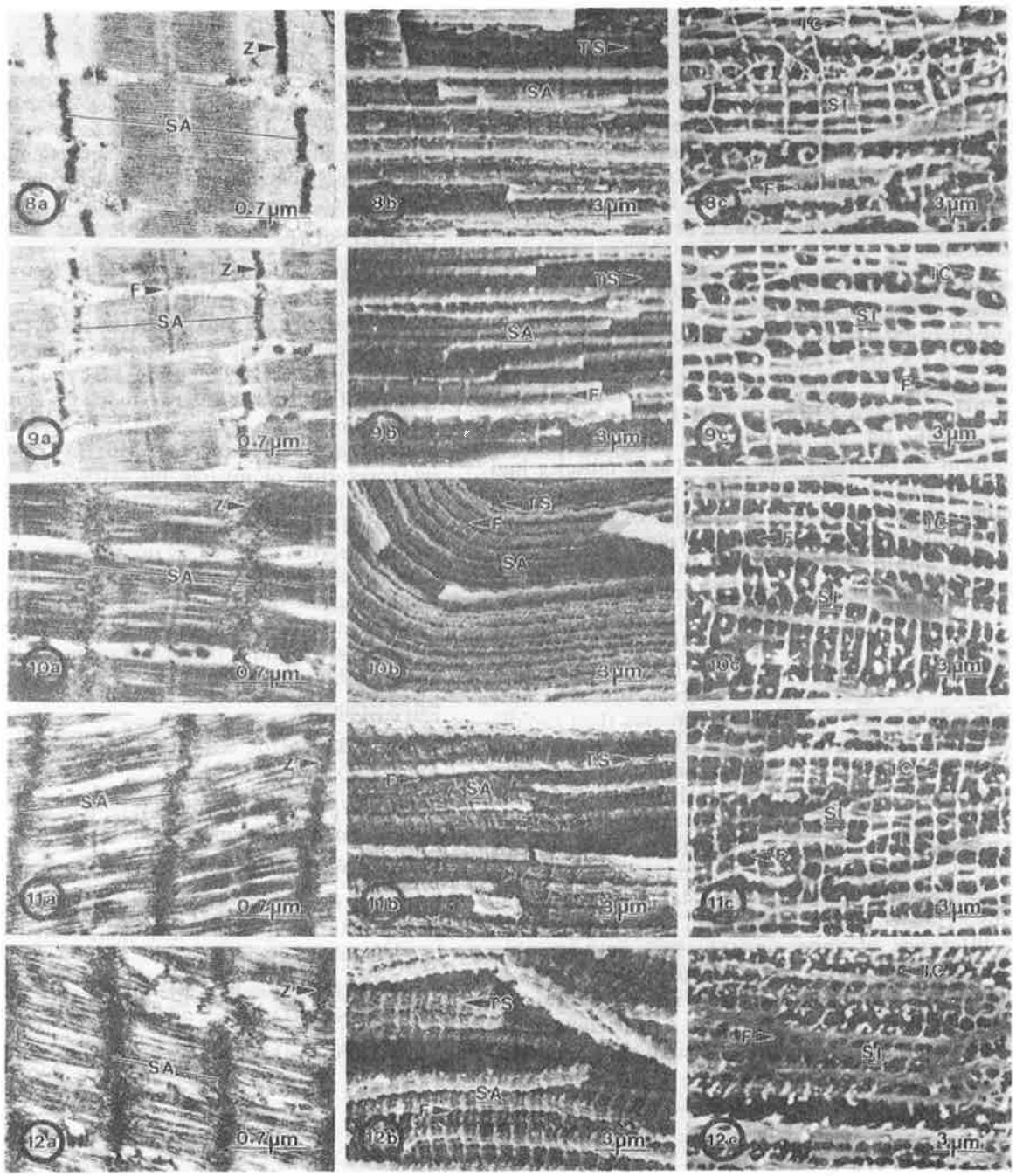

Figure 3. Electron micrographs of longitudinal sections of triceps brachii muscle using (a) transmission electron microscopy; (b) regular; and (c) scanning electron microscopy: (8) pre-rigor control; (9) post-rigor control; (10) 2°C/12 min, 47°C; (11) 2°C/12 min, 53°C; (12) 2°C/2 min, 53°C. Z-disk (Z), sarcomere length (SA), transverse structure (TS), intermyofibrillar connection (IC), spacing between intermyofibrillar connections (SI) and small filament (F).

tissue. Micrographs 7a and 7b represent muscle that was conventionally fixed and observed by TEM. Micrograph 7a shows excellent preservation of myofibrils and other structures. This method permits morphological studies and quantitative measurements of SA. Micrograph 7b shows an adequate preservation of myofibrils, but interpretation of the endomysium between muscle cells is difficult.

Correlative Microscopy. Micrographs 8a, 8b, 8c illustrate ultrastructure of myofibrils in prerigor muscle. SA was between 2.49 lm (LM and SEM-RS) and 2.71-2.77 lm (SEM-CS and TEM). There is an average SA difference of 11% between TEM and LM or SEM-RS and 9% between SEM-CS and LM or SEM-RS. Micrographs 9a, 9b, 9c illustrate ultrastructure of myofibrils in postrigor muscle. These muscles had longer ($P<0.05$) sarcomeres than those subjected to heating. Moreover, there is an average SA difference of approximately 28.8% between postrigor and prerigor muscles which is consistent with 27.9% muscle shortening. SA and spacing between IC were similar ($P<0.05$) among all electron microscopy preparation techniques. Micrographs 10a, 10b, 10c illustrate ultrastructure of myofibrils in slowly heated (2°C/12 min to 47°C) prerigor muscle. These muscles had significantly longer ($P<0.05$) sarcomeres than rapidly heated (2°C/2 min) muscles. In addition, there is an average SA difference of approximately 40% between this treatment and prerigor control muscles, which is consistent with the observed 41.3% muscle shortening. Micrographs 11a, 11b, 11c illustrate ultrastructure of myofibrils in slowly heated (2°C/12 min to 53°C) prerigor muscle. These muscles had SA similar ($P<0.05$) to that of rapidly heated muscles using LM. There is an average SA difference of approximately 48% between this treatment and prerigor control muscles which is slightly different from the observed 42.0% muscle shortening. Micrographs 12a, 12b, 12c show ultrastructure of myofibrils in rapidly heated (2°C/2min to 53°C) muscle. These muscles developed the highest degree (P.05) of shortening and the shortest SA as compared to other heating rates. In addition, there is an average SA difference of approximately 57% between rapidly heated and prerigor control muscles, which is consistent with the observed 56.2% muscle shortening.

CONCLUSIONS

Micrographs from SEM-CS (high ice sublimation, cryoprotectant), SEM-RS (fixative and freeze-fracturing) and TEM were appropriate for exposing detail of inter- and intracellular structures, for quantitative measurement of sarcomere length and spacing of intermyofibrillar connections and for minimal artifact formation. The data collected and electron micrographs viewed explain the controversial interpretation of muscle ultrastructure in cold and regular stage SEM. Sarcomere length and spacing of intermyofibrillar connections were similar ($P<0.05$) among electron microscopy preparation treatments and both change similarly across heating treatments. These results suggest that the transverse structure viewed by SEM-RS and the intermyofibrillar connections viewed by SEM-CS are found at the same sarcomere locations and are probably Z-disks.

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