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An Improved Biopsy Technique for Light and Electron Microscopic Studies of Human Skeletal Muscle*

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Skeletal muscle is one of the most surgically accessible tissues in the body, yet histologically adequate muscle biopsies are not easy to obtain owing to the contractile quality of this tissue. Both surgical manipulation and contact with the fixing solution excite fresh muscle fibers to contract, often resulting in a curled, twisted piece of tissue from which it is hard to obtain properly oriented sections. Several instruments have been devised with the object of overcoming this difficulty in handling muscle tissue; however, they have generally proved to be too cumbersome for careful surgical technique, and some were quite painful to the patient.¹ The most satisfactory methods^{1, 15} for handling biopsy material that have been employed to date involve carefully placing a small straight piece of tissue on cardboard (sometimes slightly stretching the specimen) and then placing it in a Petri dish containing filter paper moistened with normal saline solution for 15 to 30 minutes prior to immersing it in fixative. Although this procedure has usually proven to be satisfactory for conventional light microscopic evaluation, it is a time-consuming and annoying chore for the busy physician

or technologist. In addition, where electron microscopic studies are anticipated, this method is not conducive to good cell preservation. In order to overcome these handicaps, we have devised a new biopsy procedure that has been used successfully thus far in 30 patients to obtain well-fixed, uniform, interpretable tissue sections for either electron microscopic or light microscopic evaluation.

BIOPSY

The biopsy procedure is primarily dependent on the use of a new surgical clamp† that we have devised (Fig. 1). This clamp is in essence a modified forceps with a handle made of the usual surgical spring steel, and a two-pronged fork tip of a firmer, highly corrosion-resistant stainless steel that is not chemically affected by the fixative osmium tetroxide. It has a setscrew at the midpoint of the handle which makes it possible to permanently retain the specimen and prevent it from retracting once it has been clamped and dissected free.

It is also surgically advantageous to fashion a thin double-edged razor blade into a scalpel blade by breaking it in two and rebreaking each half at a 45° angle. Mainly because of its flexibility coupled with its sharpness, the resultant blade when grasped with a small hemostat makes a more satisfactory tool for sharp delicate dissection than most standard surgical scalpel blades. It must be borne in mind, however, that all razor blades have a thin coating of grease that may interfere with the effectiveness of the fixative; therefore,

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† Patent pending.

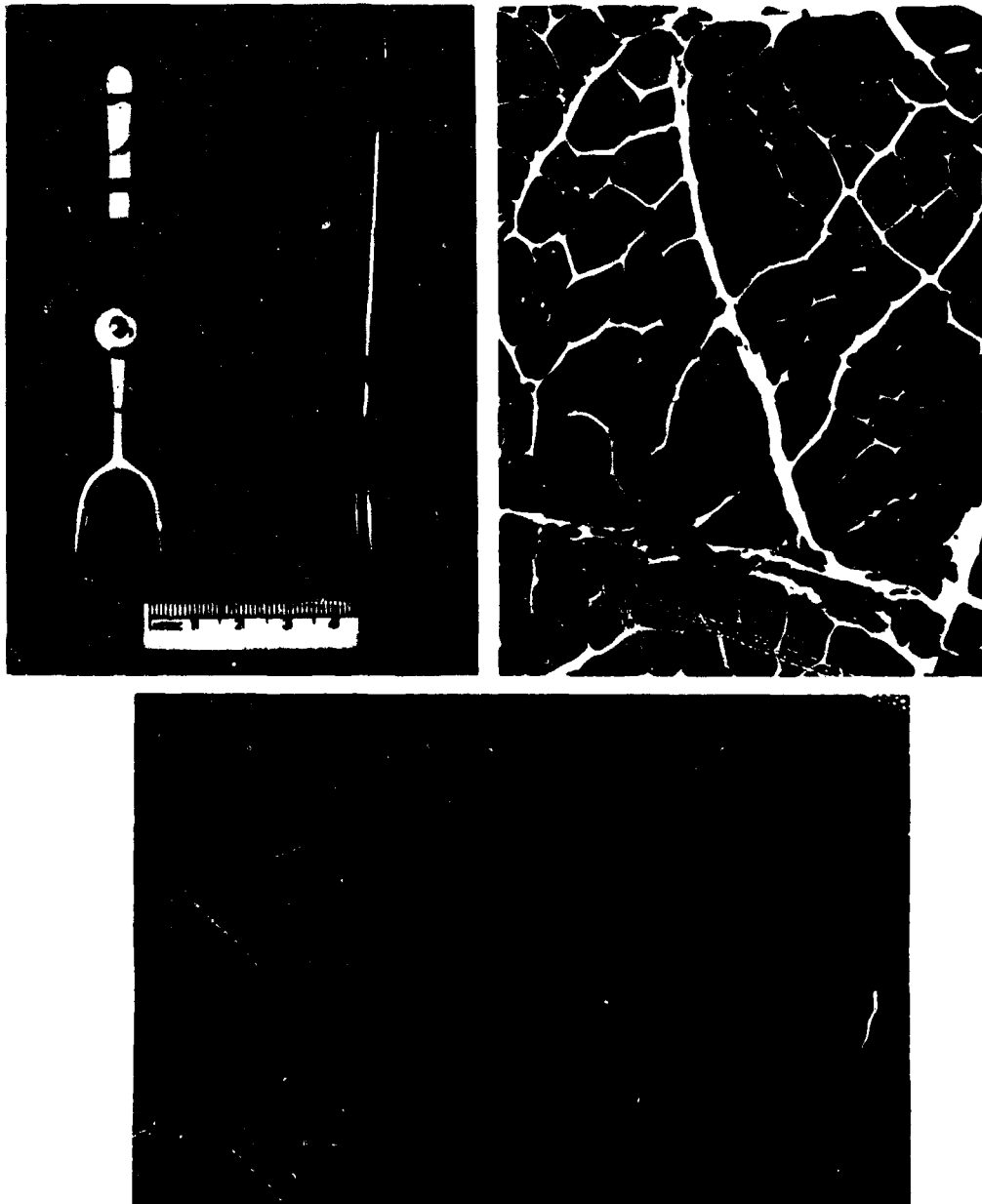


FIG. 1. Muscle biopsy clamp. The body of the clamp is made of the usual surgical spring steel. The two-pronged fork tip is made of a firmer, highly corrosion-resistant stainless steel that will resist the chemical effects of the fixative O_3O_2 . Clamps with widths of 0.5 to 2.0 cm. between the fork tips have been used.

FIG. 2. Human quadriceps femoris. Cross-section. Fixed in 6.5 per cent glutaraldehyde solution diluted with Millonig's buffer.² Light micrograph. Hematoxylin and eosin (H&E); $\times 480$.

FIG. 3. Human quadriceps femoris. Longitudinal section. Clamped and then fixed in 10 per cent neutral formalin solution. Even the Z-line can be readily delineated in this routinely fixed and stained surgical biopsy. Light micrograph. H&E; $\times 480$.

prior to sterilization the razor blades should be briefly rinsed in acetone to remove this packing lubricant. Sterilization should be by soaking in a germicide solution rather

than autoclaving, since that dulls the fine sharpness of the blade.

In taking biopsy specimens from young children, it is often desirable to use a gen-

eral anesthetic or regional block; however, for most biopsies from older children and adults, a locally infiltrated anesthetic is satisfactory, providing the patient has been premedicated. The surgeon must be cautioned not to inject the local anesthetic agent into the muscle specimen to be biopsied. On exposing the body of the muscle to be biopsied, the overlying fascia is carefully dissected free from the underlying muscle. A narrow strip of muscle fasciculi is developed by making two parallel incisions in line with the muscle fibers. A third incision inferiorly leaves this slip of muscle attached only at its ends. The loosened fascicular strip is then grasped with the fork tips of the clamp and the screw of the clamp is promptly tightened, holding the muscle fibers at their original length. The clamped muscle segment is freed from the body of the muscle by sectioning along the outer margin of each fork tip of the clamp.* Care is taken to avoid any unnecessary manipulations of the biopsy specimen. The clamp tip with the secured specimen is then immediately immersed and gently agitated in a container of fixative to flush away any excess serum or blood, and then placed in the final fixative solution. The most desirable specimens for light microscopy are found to be 1.5 to 2 cm. long, 1 cm. wide, and 0.5 to 0.8 cm. thick; for electron microscopy, 1.0 to 1.5 by 0.5 by 0.5 cm.

FIXATION AND STAINING

ELECTRON MICROSCOPY

Both 2 per cent osmium tetroxide and 2 to 6.5 per cent glutaraldehyde solutions diluted with Millonig's buffer⁹ have been used as fixatives. The tissue is left in the fixative with the clamp still applied for approximately 15 minutes. Small blocks (2 by 0.8 by 0.8 mm.) of tissue are then cut

* Occasionally it has been found convenient to vary the biopsy technique as follows. After the two parallel incisions have been made, the clamp can be applied vertically from above and tightened on the muscle strip. One end of the strip is then cut outside the clamp, which is then used as a gentle lever to elevate and expose the under surface of the strip for dissection from the underlying muscle mass. Finally the distal end of the strip is divided and the biopsy specimen removed.

from *margins* of the large biopsy specimen, taking care to orient the muscle fibers and myofibrils parallel to the long axis of each block. These pieces of tissue are kept moist with the fixative during the trimming process and afterwards they are placed back into the original container of fixative until fixation time is complete. Fixation time for OsO_4 has been 45 minutes at room temperature, and for glutaraldehyde, 4 hours at 4° C. After appropriate washing in Millonig's buffer,⁹ the tissue fixed in glutaraldehyde is postfixed with buffered OsO_4 .¹² The tissue samples are then dehydrated and embedded in one of the epoxy resins, usually Araldite.⁸ Some of the tissue blocks are stained with 1 per cent phosphotungstic acid⁸ prior to embedding (Fig. 4). Tissue sections on the grids are stained with uranyl acetate¹⁴ (Figs. 7 and 8) or lead citrate,¹¹ or both (Figs. 5 and 6).

The irritant effect of the fixative, especially OsO_4 on excitable muscle tissue usually causes some pulling against the rigidly fixed clamp margins. Specimens obtained from these regions may have slightly overstretched sarcomeres. Markedly contracted sarcomeres are rare. Usually the most satisfactory OsO_4 material is that obtained from the middle third of the biopsy specimen.

As compared to OsO_4 , glutaraldehyde seems to be less of an irritant to freshly excised muscle as well as a better fixative for the contractile filamentous organelles (actin and myosin) (Figs. 6 to 8). The band pattern of the myofibrils is more clearly defined, and actin and myosin filaments are found to have a larger diameter than OsO_4 fixation alone has indicated. Connecting bridges between the thick myosin filaments in the M zone, which have only been vaguely demonstrated in heart¹³ and skeletal¹³ muscle fixed with OsO_4 are more readily delineated in the glutaraldehyde preparations.† In some cross-sections, the myosin filaments even exhibit a hollow center (Fig. 7). Recent studies of fish³ and rabbit⁷ skeletal muscle fixed with glutaraldehyde concur with these observations of the myofibrils. In addition, the diameters of

† These structures are distinct from the cross bridges between thick and thin filaments as first described by Huxley⁴ in the regions of the A-band where the thick and thin filaments interdigitate.



FIG. 4. Human quadriceps femoris. Longitudinal section depicting segments of several myofibrils. The components of each sarcomere are readily identifiable. Note their uniformity in length. For details of the normal ultrastructure of skeletal muscle the reader is referred to several review articles.^{1, 2, 3} Fixed in 2 per cent solution of OsO_4 diluted with Millonig's buffer.⁴ Tissue blocks were immersed in 1 per cent phosphotungstic acid solution of absolute alcohol before embedding in Araldite.⁵ No other staining was done. Electron micrograph. $\times 19,000$.

FIG. 5. Human quadriceps femoris. Longitudinal section showing tubular components of the sarcoplasmic reticulum. Tissue fixed and embedded as in Figure 4; however, the tissue section was stained with uranyl acetate⁶ and lead citrate⁷ in order to make it easier to identify the membranes defining the sarcoplasmic reticulum and mitochondria. T, Specialized region of the sarcoplasmic reticulum referred to as the "triad." Electron micrograph. $\times 32,000$.

negatively stained *isolated* thick filaments which have not been fixed with OsO_4 have been found to be larger than material fixed in OsO_4 .⁸

The morphologic characteristics of glycogen also appear to be changed by glutaraldehyde fixation. In tissue fixed only with

OsO_4 , the glycogen granules are not as readily stained with uranyl acetate as with lead solutions. In the glutaraldehyde preparations, however, uranyl acetate alone very effectively stains the glycogen granules and, in addition, these granules have a slightly larger average diameter.

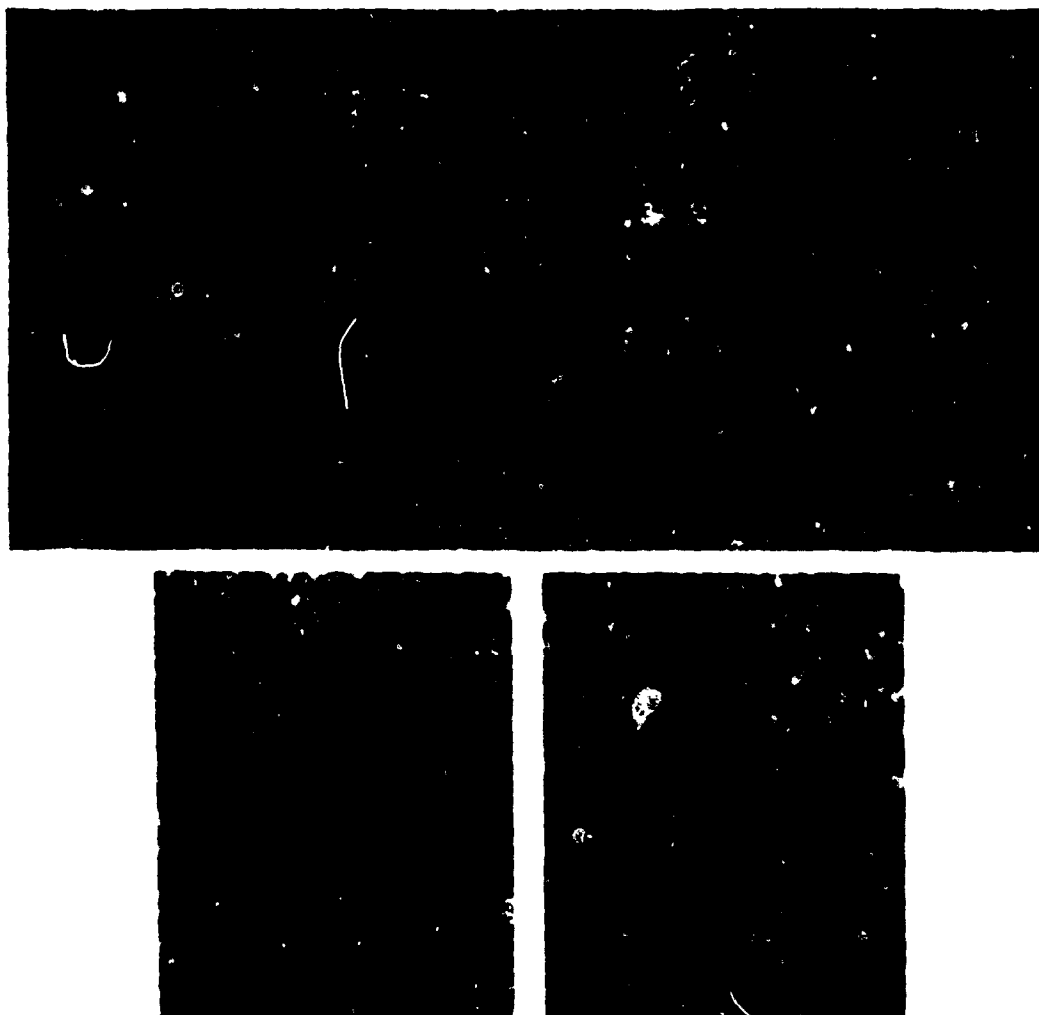


FIG. 6. Human quadriceps femoris. Longitudinal section revealing the numerous glycogen particles which abound predominantly in the intermyofibrillar spaces. The mitochondria are typically disposed in pairs opposite the I-bands. Fixation in 6.5 per cent glutaraldehyde solution diluted with Millonig's buffer² and postfixed in 2 per cent OsO₄, prepared with the same buffer. Tissue blocks were stained with phosphotungstic acid (PTA) (as in Fig. 4) and tissue section was stained as in Figure 5. The myofilaments and glycogen particles are better preserved than in OsO₄ fixed material. Electron micrograph. $\times 22,000$.

FIG. 7. Human quadriceps femoris. Cross-section through the A-band of a myofibril depicting the interdigitating thick and thin filaments which make up this region of a myofibril. Each thick filament is surrounded by six thin filaments. An occasional myosin filament is seen which has a prominent hollow center (*arrow*). Tissue fixed as in Figure 6. Tissue blocks were stained with PTA, and tissue section was stained with uranyl acetate. Electron micrograph. $\times 130,000$.

FIG. 8. Human quadriceps femoris. Cross-section through M-line region of H-zone of a relaxed sarcomere. No thin (actin) filaments are present in this region; only thick (myosin) filaments are present, and these have a slightly increased diameter in this zone of the sarcomere. They are interconnected by discrete cross bridges which are more readily delineated following fixation with glutaraldehyde. Tissue stained as in Figure 7. Electron micrograph. $\times 160,000$.

LIGHT MICROSCOPY

Specimens may be fixed in either glutaraldehyde or neutral formalin solutions (Figs. 2 and 3). With these larger pieces of

tissue the clamp is left on for a longer time (about 45 minutes). The tissue is then cut from the clamp and readily sectioned into proper longitudinal and cross-sections.

Hematoxylin and eosin or special stains are employed after the usual methods of paraffin embedding.

COMMENTS

The size of the clamp allows one operator to easily manipulate it. The delicate nature of the procedure is implied by the simple design of the clamp, the need for clean, sharp dissection, and the inherent irritability of viable muscle fibers. Sharp dissection is best attained if the surgeon uses the double-edged razor blade, as suggested above. The design of the instrument practically precludes securing a poorly oriented specimen since it can only be successfully applied to carefully exposed longitudinally oriented muscle fasciculi; therefore, it is quite simple for the pathologist to cut well-oriented longitudinal and cross-sections and also avoid the crushed edges of the specimen. During the entire initial steps the muscle is kept under reasonable natural tension so that artifacts secondary to marked contracture or overstretching are avoided. In conclusion, a more uniformly satisfactory muscle biopsy specimen can be obtained for both light and electron microscopic examination by the techniques as outlined in this brief report. It is reasonable to suggest that such a technique should become standard procedure in order to avoid the handling artifacts that otherwise invariably occur in viable muscle and which have made precise histologic interpretation of muscle biopsy material extremely difficult.

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