Needle muscle biopsy in the investigation of neuromuscular disorders

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ABSTRACT: We have evaluated needle muscle biopsies in 220 patients with various neuromuscular disorders, using a method developed previously at Tuft's University. The method uses a 14-gauge needle propelled by an automatic device. An average of 3.5 samplings were taken per patient. Muscle samples were used for histological and molecular genetic analysis, and for the isolation of muscle satellite cells for in vitro cultures. The biopsy is well tolerated by the patients who never declined multiple samplings. Complications were few and minor, with no sequelae. In most cases the small size of the muscle specimen (ca. 15 mg per sampling) was sufficient to perform the various procedures and to yield a diagnosis. Specimens were considered insufficient for histological results in 9 patients (4%), due to technical artifacts or insufficient material. We now routinely use this method, which has several advantages over the surgical technique for most muscle biopsies. © 1998 John Wiley & Sons, Inc. *Muscle Nerve* **21:** 194–200, 1998
Key words: human muscle culture; mitochondriopathy; myoblasts; myopathy; myositis

NEEDLE MUSCLE BIOPSY IN THE INVESTIGATION OF NEUROMUSCULAR DISORDERS

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Muscle biopsies have been used as a diagnostic procedure in neuromuscular disorders since 1865.[7,10] Surgical biopsies are time-consuming and require general anesthesia in children. Needle muscle biopsies (NMB) do not have these drawbacks (review in Refs. 9, 13, and 17). Several types of needles have been used, but this practice has not gained a wide acceptance, allegedly because of inadequate size of specimens. In spite of these limitations we have evaluated a method developed previously at Tuft's University.[8] We report histological and genetic findings in 220 patients, and show that it is possible to recover the satellite cells from the small biopsy to prepare muscle culture.

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PATIENTS AND METHODS

Patients.

NMBs were performed from January 1994 to November 1996 in patients with various neuromuscular disorders as suspected by clinical presentation, electrophysiology, and blood tests. Altogether 220 patients were biopsied by this method (64 patients in 1994, 76 in 1995, and 80 in 1996).

Muscle Biopsy Technique.

Details concerning the technique have been described by Coté et al.[8] Briefly, after skin sterilization and local anesthesia using 1–2 mL of xylocaine 2% subcutaneously, a 1–2-mm-long skin cut is made with the tip of a scalpel. The patient is asked to contract the muscle to be biopsied. A 14-gauge disposable needle (Bard biopty-cut, 160 mm) is then inserted through the cut, perpendicular to the muscle fibers, until the fascia is pierced. The automatic biopsy device (Bard biopty

Instrument, Bard Radiology, Covington, GA) is triggered. In less than 100 ms the core of the needle that carries a sampling notch penetrates (length of the notch 16 mm, course 23 mm), followed by a tubular knife which cuts the muscle, leaving a small sample in the notch. Several samples may be collected from one puncture site. Muscles commonly biopsied were quadriceps (vastus lateralis), deltoideus, and tibialis anterior. A local compression on the biopsy site is then applied for 10 min, an adhesive bandage is applied, followed by an ice bag. The patient may walk after 2 h rest. Contraindications to the procedure are anticoagulation or blood dyscrasia. Obtaining samples from severely wasted muscles may be difficult. This difficulty may be minimized either by directing the needle as close as possible to the femur bone (in the case of quadriceps biopsy) or by choosing a less diseased muscle when available.

Specimen Preparation and Evaluation. The muscle samples are removed from the needle using the blade of a scalpel, and they are carefully placed on a gauze moistened with NaCl 0.9%. Within a few minutes the samples are oriented under a stereomicroscope in such a way as to obtain appropriate transverse sections of the fibers. Samples are then transferred onto a layer of tragacanth gum (Sigma) spread over a slice of cork with care being taken to avoid tissue fragmentation. The specimen is frozen on this support in a beaker containing 2-methylbutane (Merck) immersed in liquid nitrogen and then transferred to a freezer at −80°C. The following stains are routinely performed: hematoxylin and eosin, Van Gieson Elastin, periodic acid-Schiff, modified Gomori trichrome, Nile Blue, reduced nicotinamide adenine dinucleotide tetrazolium reductase, adenosine triphosphatase (pH 9.4, 4.6, 4.3), and cytochrome *c* oxydase. Immunostains can be performed on the same material to detect dystrophin, adhalin, merosin, or inflammatory cell subtypes. A single needle biopsy yields up to 1000 muscle fibers. It is noteworthy that the edges of the samples show only few traumatic artifacts with respect to the sampling procedure. Fragmentation of the material may occur with handling, or possibly due to the fragility of the tissue caused by the underlying muscular pathology. For a current histological analysis three samples of good quality (of 8–12 mm in length) are sufficient. Ultrastructural examination and/or biochemical investigation require additional samplings. Electron microscopy is not tested in this series; it could be done, but tissue cannot be fixed at resting length.

Genetic Analysis. DNA was prepared from single biopsies or from fragments unsuitable for histological analysis. Total DNA (mitochondrial and nuclear) was extracted from approximately 10–15 mg of tissue, by proteinase K digestion and phenol/chloroform extraction. Samples were referred for screening for common mitochondrial DNA (mtDNA) mutations. Deletions and duplications were sought by Southern blotting, using the restriction enzymes BamH1 or Dra1 and purified human mtDNA as probe. Three relatively common mtDNA point mutations (at positions 3243, 8344, and 8993) were analyzed by polymerase chain reaction (PCR) amplification using mismatch primers followed by restriction digests. In addition, two frequently mutated mitochondrial transfer RNA (tRNA) genes ($tRNA^{Leu(UUR)}$ and $tRNA^{Lys}$) were screened for further mutations by single-stranded conformation analysis (SSCA). Briefly, the two genes plus approximately 100 base pairs of flanking sequences were amplified by PCR; the produces were heat-denaturated in 95% formamide, and migrated on a Pharmacia Multiphor II through 12.5% Excelgels for 2400 volt-hours at 15°C. Bands were visualized by silver-staining.

Cell Culture. Human muscle satellite cells were isolated from NMBs by adapting the procedure described by Blau and Webster[5] and recently modified by Baroffio et al.[1] The sample is rinsed in a solution containing: glucose 10 mmol/L, NaCl 130 mmol/L, KCl 3 mmol/L, NaH_2PO_4 1 mmol/L, Hepes 30 mmol/L, pH 7.55. The tissue is weighed and put in a 35-mm Falcon petri dish containing 2 mL trypsin–edetic acid (EDTA) (0.05%–0.02% in the solution described above). The dish is then placed in an incubator for 1 h at 37°C. During the first half hour of incubation the dish is agitated manually three times. Muscle fragments are then transferred to 2 mL of proliferation medium (see below) to remove trypsin–EDTA and block proteolytic activity. Fragments are gently triturated using Pasteur pipettes of decreasing diameters. At this stage, the isolated satellite cells in the suspension are visible under a microscope. The suspension is centrifuged at 400 rpm for 2 min in order to eliminate the majority of muscle debris. Single satellite cells in the suspension are then manually collected with a micropipette under a microscope and are transferred to a dish in which each cell is cultured as a clone in proliferation medium. This technique ensures that a single cell is put in each culture dish. Proliferation medium is made up of F10-Gibco nutrient medium supplemented with 15% fetal calf serum, 0.5 mg/mL bovine serum

albumin, 0.5 mg/mL fetuin, 10 ng/mL epidermal growth factor, 0.39 mg/mL dexamethasone, 0.18 mg/mL insulin, and 0.1 µg/mL gentamycin.[11] In this medium, activated satellite cells become myoblasts and divide actively. When confluent, cells are replated at a lower density. Half of the proliferation medium in the cultures is replaced three times a week. Clonal cultures of myoblasts can proliferate for 2–3 months.[19] Formation of myotubes can be induced by replacing the proliferation medium with a medium that promotes myoblasts differentiation and fusion.[18] The differentiation medium is made up of Dulbecco's modified Eagle's medium (DMEM)–Gibco nutrient medium supplemented with 0.5 mg/mL bovine serum albumin, 10 ng/mL epidermal growth factor, 10 µg/mL insulin, and 1 µg/mL gentamycin. Half of the culture medium is changed three times a week. Proliferating myoblasts derived from a single satellite cell are illustrated in Fig. 1 (left). After 3 days in differentiation medium many myotubes are present in the cultures (Fig. 1, right).

RESULTS

Two hundred and twenty patients (101 men, 110 women and 9 children) aged 2–88 years (average 47 years) were biopsied. In 1 child, a NMB was performed during a general anesthesia required for magnetic resonance imaging (MRI); 3 children received a light sedation (as performed by others[13]); 5 children aged 10 years and over received no sedation and were able to cooperate. Muscles sampled were: quadriceps (vastus lateralis) $n = 198$, deltoideus $n = 27$, tibialis anterior $n = 9$. Number of samplings per patients was on average 3.5 (extrema 1–6). Complications were: hematoma $n = 2$, syncope $n = 1$; sequelae $n = 0$. Pain related to the biopsy procedure, when experienced, is very brief. On some occasions a small nerve was found in the specimen. The patients sometimes mention the sensation of having been punched that lasts for up to 4 days.

Histology. A histological examination was performed in all 220 patients. It was abnormal and confirmed the clinical suspicion in 83 patients (38%); it was abnormal and yielded an unexpected diagnosis in 12 patients (5%); it showed minimal aspecific changes in 50 patients (23%); it was normal and excluded a weak clinical suspicion in 60 patients (27%); it was normal in spite of a strong clinical suspicion in 6 patients (3%). The specimen was considered insufficient for histological results in 9 patients (4%). Some histological results are given in Table 1, and examples are illustrated in Figure 2.

Genetics. One hundred muscle biopsies were subjected to molecular genetic analysis. In all cases but 1 (99%), sufficient DNA was obtained for Southern blot and PCR analysis of mtDNA. One NMB did not provide sufficient DNA for Southern blotting, but PCR analysis was possible. Although the yield was not routinely determined, it was on average between 1 and 4 µg of total DNA. Southern blot analysis of nuclear genes would not generally be possible with

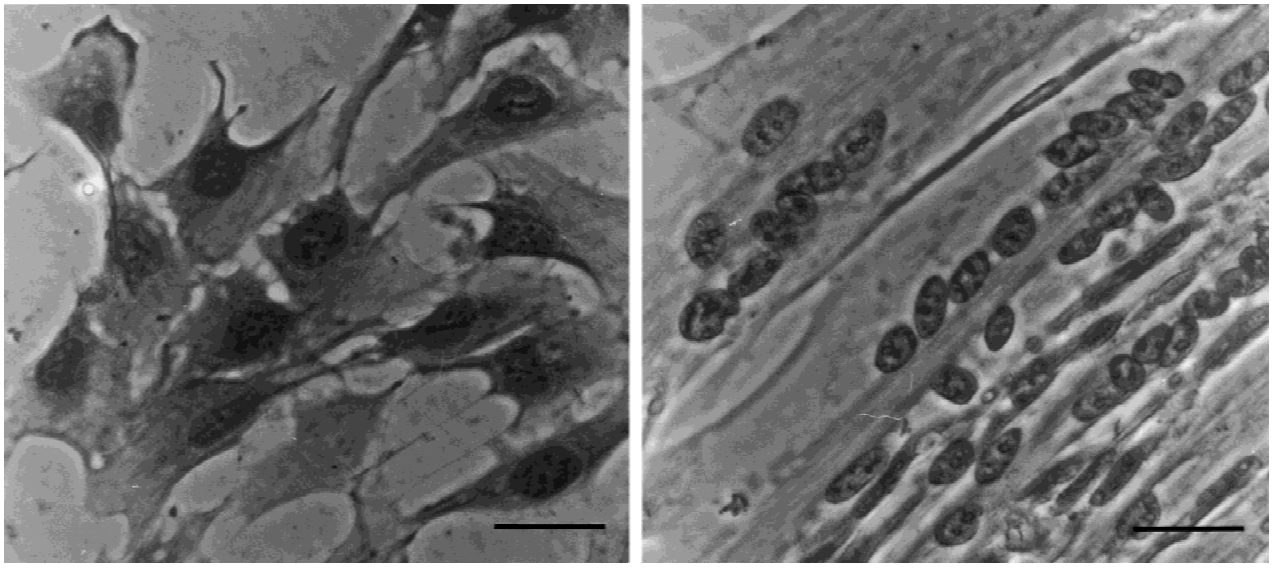

FIGURE 1. Hematoxylin-stained culture of proliferating myoblasts (left) and myotubes (right). To induce myotubes formation, the culture was kept in differentiation medium for 3 days. Bars represent 30 µm.

Table 1. Results of histology.

Normal		66
Myopathy		83
Polymyositis	20	
Sarcoidosis	2	
Intensive care myopathy	9	
Fibrosis	2	
Muscle dystrophy	5	
Lipidic myopathy	12	
Steroid myopathy	3	
Mitochondrial accumulation and proliferation	29	
Multicore myopathy	1	
Neurogenic abnormalities		27
Vasculitis		2
Aspecific abnormalities		33
Insufficient sampling or technical problem		9
Total		220

such a yield, particularly in light of the reduced nuclear/mitochondrial DNA ratio of muscle compared to other tissues.

Heteroplasmic deletions of mtDNA were identified by Southern blot analysis in just 2 patients (1 of them is illustrated in Fig. 3), and no heteroplasmic point mutations were detected. In 1 patient a novel homoplasmic sequence variant was identified in the $tRNA^{Leu(UUR)}$ gene by SSCA and sequence analysis; in the absence of a family history of neuromuscular disease, it was concluded that this was probably a nonpathogenic polymorphism (data not shown).

Cell Culture. A typical needle biopsy weighs 15 mg (n = 6) and contains approximately 1000 satellite cells. Note, however, that a single satellite cell can

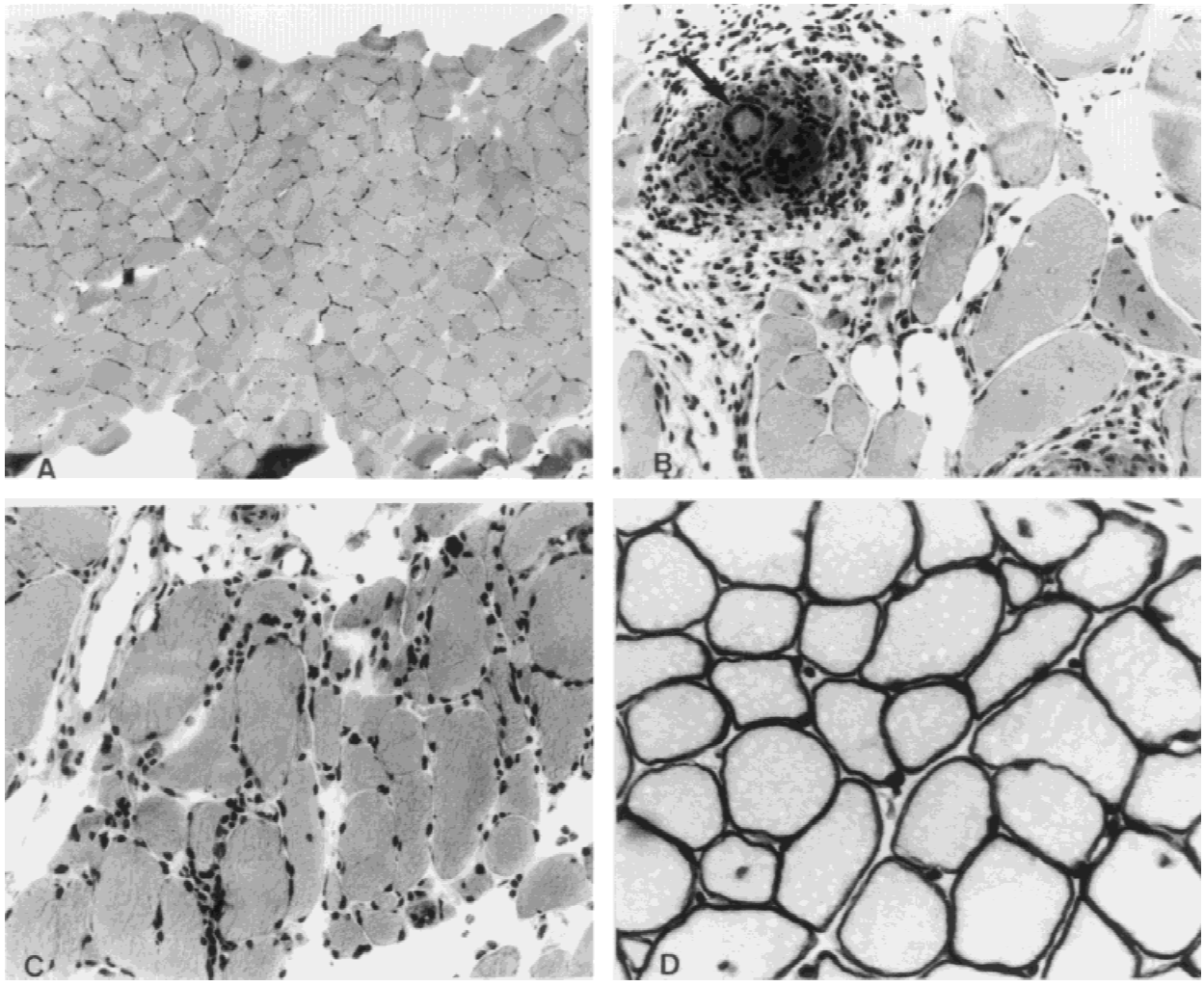

FIGURE 2. Histological aspects of needle biopsies. **(A)** Normal muscle: the picture represents about one third of a sample of good quality [hematoxylin and eosin (HE) ×63]. **(B)** Sarcoidosis: a granuloma with giant cells (arrow) in the muscle interstitial connective tissue (HE ×160). **(C)** Polymyositis: muscle fibers of variable size surrounded by lymphocytic infiltrate (HE ×160). **(D)** Merosin immunolocalization showing normal distribution of the protein (×400).

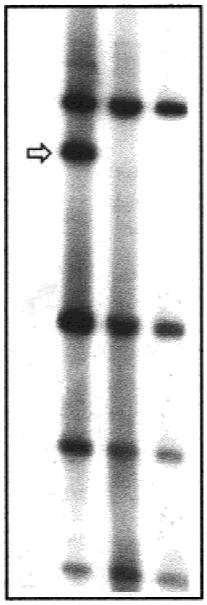

FIGURE 3. Southern blot analysis of mtDNA from representative NMBs, one with a heteroplasmic deletion and two with no mutation detected. DNA was digested with Dra1. The arrow indicates the abnormal band produced by the deletion.

divide up to 60 times in culture (depending upon the age of the donor). Thus, each satellite cell can produce many monolayer cultures. Figure 4 compares, during the first days in culture, the growth characteristics of satellite cells collected from 7 surgical biopsies (A) and 6 NMB (B). Control (surgical) biopsies weigh 496 ± 88 mg (n = 7); they were taken from patients aged 7 months to 17 years (average 7 years) undergoing orthopedic surgery. NMB were taken from patients in whom the possibility of a mitochondrial myopathy was suspected. Patients were between 17 and 65 years old (average 40 years). It can be seen that there are no major differences in the development of cells from surgical or NMB during the first week in culture. The cultures developed from fine NMB as well as the cultures from surgical biopsies and cells could be frozen for further investigations. Given these observations, it is not surprising that confluent myoblasts obtained from NMB, when exposed to differentiation medium, behaved exactly as cells obtained from surgical biopsies. Fusion of myoblasts began within 2 days and, with time in culture, some myotubes began to contract spontaneously and to present cross-striations.

DISCUSSION

NMB using the described technique can be easily performed in ambulatory patients (even in children) and proves safe. It can be used to obtain samples from more than one muscle in a given patient, and can be more easily repeated than surgical biopsies. Pain is not a concern, and the advantage of the propelling device used, over other types of NMB, is that biopsy is performed extremely rapidly. The technique is well tolerated by the patients who never declined multiple samplings. Only minor complications were encountered with no sequelae, and almost no scare. The biopsy may be performed by the neurologist, as was done in our study (M.R.M.; A.K.). It yields muscle specimens that are sufficient for histologic examination, as also stated by others.[8,9,13,17] It further allows molecular genetic interpretations and myoblast cultures that are comparable to surgical procedures, as shown in this study. The small sampling size is somewhat counterbalanced by the possibility of obtaining several samplings from different areas of the muscle. This proved particularly useful in myositis and sarcoidosis. If required, the best

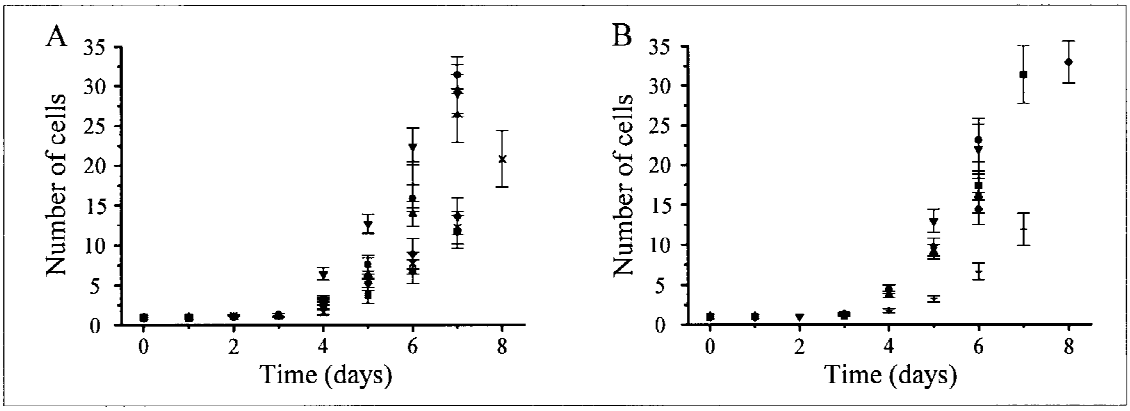

FIGURE 4. Number of myoblasts as a function of time in culture: a comparison between surgical **(A)** and needle **(B)** muscle biopsies during the first days after dissociation. Between 7 and 18 clonal cultures were evaluated for each biopsy. Each biopsy is represented by a different symbol.

sampling site may be guided by MRI. In case of an unsatisfactory result, or of a normal finding when a strong clinical suspicion exists, the NMB may be repeated, or a surgical biopsy may be performed. In this series of 220 patients, repetition of NMB has been performed in 5 patients, and a surgical biopsy in 2 patients. Repetition was required four times because of insufficient material (once due to a hematoma) and eventually yielded a diagnosis (1 normal; 1 muscular dystrophy; 2 lipidic myopathies); on three occasions repetition did not change the result (2 normals and 1 myositis).

Histologic Examination. NMB requires dedicated collaboration of the neuropathologist, and rapid orientation of the biopsy under a stereomicroscope is essential. Conventional staining is then possible in the same manner as surgical biopsies. In spite of the small size of the biopsies, fragments have been frozen for further analysis.

Genetics. The diagnostic yield of the molecular analyses is low in this series (only two heteroplasmic deletions of mtDNA detected in 100 biopsies tested). This may be partly due to the fact that the ease of the NMB procedure allows us to biopsy patients with milder symptoms or earlier in their disease course. Alternatively, it is possible that the molecular screening protocol is insufficiently sensitive, or that NMB at a single site is not suitable when a heteroplasmic mutation is suspected. In this respect, we suggest that a second NMB be considered when no mtDNA mutation is detected in a strongly suspect patient.

Cell Culture. Muscle cell culture has demonstrated its value in the study of muscle physiology. In vivo, muscle satellite cells are quiescent mononucleated cells lying on skeletal muscle fibers.[6,16] In response to various stimuli, these cells can proliferate as myoblasts and fuse, thereby contributing to growth, repair, and hypertrophy of postnatal skeletal muscle. It is also the satellite cells recovered from a muscle biopsy that give rise to myoblasts and myotubes in culture. Our goal here was to devise a method for isolating the satellite cells present in the small muscle samples obtained from fine NMB. The possibility of controlling the degree of maturation of cultured human muscle cells using different culture media[11,18] and the possibility of identifying and purifying human muscle satellite cells immediately after dissociation of a muscle biopsy[1] have opened new perspectives. It is now possible to study physiological and physiopathological properties of satellite cells[2,12] and of their progeny[3,4,15] in pure cultures that are not ''contaminated'' by nonmuscle cells. In view of this, the method described here for recovering satellite cells from fine NMB should be useful for the exploration of various aspects of muscle disorders in cell culture. Future development may be in the direction of muscle grafts and gene therapy.

We are currently evaluating the feasibility of biochemical analysis of the mitochondrial respiratory chain performed on these small muscle samples obtained by NMB. Although we did not test it, Western blotting should be possible with a protein extract from the small NMB sample. Dystrophin, for example, which represents 0.002% of total muscle protein, should be detectable according to published data.[14]

To conclude, NMB is now our method of choice for the majority of diagnostic muscle biopsies.

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