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REVIEW ARTICLE

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Recommendations for the biopsy procedure and assessment of skeletal muscle biopsies

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Abstract Muscle biopsy has a valuable diagnostic role in many neuromuscular diseases, but it is an invasive investigation that should not be undertaken lightly. Furthermore, the biopsy procedure and subsequent laboratory processing of the specimen may significantly influence the results. The following guide is designed to optimise the diagnostic information that can be obtained from muscle biopsy. It covers the whole procedure, from selection of the biopsy site and technique of biopsy, progressing through freezing of the specimen for histochemistry, and the role of immunocytochemistry and electron microscopy. Finally, the contents of a model biopsy report are considered.

Key words Muscle biopsy · Neuromuscular disorders · Biopsy interpretation

Introduction

The introduction of enzyme histochemistry as a routine laboratory procedure heralded a new era in the diagnosis of muscular disorders, and the more recent explosion of molecular biology continues to elucidate their pathogenesis. Muscle biopsy provides valuable diagnostic information in many patients with neuromuscular disease, but it is not an appropriate initial investigation. Histopathology should always be interpreted against the background of the clinical history and examination and results of less invasive procedures, including serum creatine kinase (CK) estimation, the electromyogram (EMG) and imaging.

Who should have a biopsy?

There are no hard and fast rules, and each case must be considered on its merits. In general, the likelihood of obtaining useful diagnostic information correlates with firm evidence of neuromuscular disease, i.e. positive clinical signs, biochemical or electrical evidence. Muscle biopsy is valuable in establishing the diagnosis of a wide range of disorders, both primary myopathies (including dystrophies, metabolic and inflammatory myopathies) and also denervating diseases, but the patient who complains of vague aches and pains and has a normal CK and normal EMG invariably has a normal biopsy. Biopsy is almost the only way of categorising the great majority of congenital myopathies [5]. A baseline biopsy is advisable before commencing steroid therapy for a suspected inflammatory myopathy. At a later stage it may be impossible to distinguish between incompletely resolved polymyositis and steroid-induced myopathy. There are, however, disorders in which the results of other tests may obviate the need for muscle biopsy.

The number of neuromuscular diseases in which the genome has been cloned and mutations identified increases almost weekly [8]. Thus, in any patient with a family history, or whose phenotype suggests a well-characterised hereditary disorder, the availability of a genetic test on a blood sample should be questioned before proceeding to biopsy. Duchenne and myotonic dystrophy and spinal muscular atrophy are three of the more common disorders in which this is now possible [4, 7, 10]. Muscle biopsy is often employed in diagnosis of mitochondrial cytopathies and the presence of ragged red and cytochrome oxidase (COX)-negative fibres may be informative, but a significant number of these disorders are due to maternally inherited mutations in the small mitochondrial genome and can be detected in leucocytes [6, 9].

Muscle biopsy is not appropriate in suspected myasthenia gravis or the myasthenic syndromes, where electrophysiological recording and acetyl choline receptor antibody detection are first-line investigations.

Value of biopsy

Muscle biopsy remains the key investigation leading to a specific diagnosis with prognostic implications and in-

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creasing potential for therapy in many acquired inherited and neuromuscular diseases. Even when histopathological changes do not provide the final answer they can direct a line of further biochemical investigation and/or genetic analysis of the biopsy.

Advice to the clinician

Role of biopsy

The neuropathologist should advise on the value of biopsy in different neuromuscular disorders e.g. biopsy is not appropriate in the first instance when the clinical diagnosis is myasthenia gravis; it is unnecessary to submit a young boy with suspected Duchenne muscular dystrophy to biopsy unless a negative result has been obtained from dystrophin gene analysis.

Biopsy site

The most appropriate site for biopsy depends on the extent and distribution of any muscular weakness. Many disorders show selective involvement of different muscle groups, and muscles without definite clinical involvement may show normal histology; for example, a deltoid biopsy is often not appropriate in facioscapulohumeral dystrophy. Equally, in progressive wasting disorders a severely atrophic muscle should be avoided. In general a moderately affected muscle should be chosen. In symmetrical disorders EMG performed on the opposite limb may give guidance. CT and MRI scanning are also valuable in this respect. Biopsy at the site of recent insertion of EMG needles or any intramuscular injection given should be avoided.

The biopsy should be taken from the belly of the muscle. Normal muscle close to a tendinous insertions may show fibre splitting and endomysial fibrosis that can be confused with muscular dystrophy: specimens obtained during corrective orthopaedic surgical procedures are usually not appropriate for the histological diagnosis of muscle disease, for example. In general, the diagnostic muscle biopsies should obtained from muscles in which the normal parameters are well established, i.e. the large limb muscles. Biopsy taken from unusual sites during the course of other operative procedures are invariably less informative and frequently misleading.

Clinical data

In order to examine and report upon a skeletal muscle biopsy the neuropathologist must be in possession of full clinical data, i.e. summary of the clinical history and examination, family history, relevant drug history, results of serum creatine kinase estimation and EMG findings. The pathologist must be informed of the site of the biopsy.

The specimen

Biopsy size

The size of the specimen that can be obtained will depend on the age of the patient and the chosen method of biopsy, i.e. needle biopsy or open biopsy. A tiny sample may be all that is possible in a neonate, whereas in an adult a larger specimen can easily be obtained. The pathologist should advise the clinician taking the biopsy on the optimal size of specimen required for all the investigations relevant to a particular clinical disorder.

Biopsy procedure and handling the fresh specimen

In an open biopsy the clinician should be advised to define the longitudinal orientation of the fascicles and to excise the muscle along this plane. Three small cylinders, each approximately 1–2 cm long × 0.3 cm in diameter, are usually sufficient. The specimens should be handled as little as possible, thus avoiding stretching and crushing, placed in a clean dry container and taken immediately to the neuropathology laboratory. For needle biopsies the clinician may be able to take more than one sample and if from different muscles these should labelled.

Transport to the laboratory

If the neuropathology laboratory is not on site, it will be necessary to make special provision either for the neuropathology MLSO to attend with chemicals and equipment for freezing or for rapid transport of the specimen. Ideally the specimen should be frozen immediately after excision, but provided it is not allowed to dry out a delay of up to 45 min will not adversely affect most histochemical tests. Such a delay will, however, compromise certain biochemical analyses, e.g. assay of respiratory chain enzyme assays and the specimen will not be appropriate for the specialised in vitro investigation of malignant hyperpyrexia. If the specimen is to be transported in the fresh state it should be placed a in a sealed “moist” container (Appendix A, “Transport of the muscle biopsy”).

If it is not possible to get the fresh specimen to the laboratory within a very short time a transport medium may be used, (Appendix A, “Transport of the muscle biopsy”). Whilst it is possible to perform enzyme histochemistry on such specimens, the results are much inferior to those in snap-frozen tissue. This may be the only solution for specimens being sent from abroad.

Orientation of the specimen

The muscle biopsy must first be orientated in order that true transverse sections can be obtained. Fibre size is a valuable diagnostic criterion and correlates with fibre diameter, but the latter can only be measured accurately if a true transverse section is obtained (Appendix A,

"Specimen orientation"). A dissecting microscope is essential for the orientation of needle biopsy specimens and very small open biopsy specimens.

Freezing the fresh specimen

In comparison with results of enzyme histochemistry on frozen sections, the diagnostic value of a formalin-fixed muscle biopsy is meagre. It is unacceptable to limit the product of an invasive investigation to formalin fixation and paraffin embedding, but the correct method of freezing is equally crucial to histological diagnosis. Direct immersion in liquid nitrogen used routinely for frozen sections of tissues such as breast is wholly inappropriate for skeletal muscle and will induce florid ice crystal artefact. A suitable method for freezing the muscle biopsy is given in Appendix A, "Freezing the specimen".

Formalin fixation

For the great majority of light microscopic preparations frozen sections are preferable, and if a specimen is very small this sample is best omitted. However, there are a very few antibodies that give better results with immunocytochemical staining in fixed tissue. To avoid hypercontraction a small cylinder should be allowed to relax at room temperature and in an air-tight container to prevent drying for approximately 20 min before immersion in formalin.

Staining procedures

Routine staining suggested minimum staining procedures	
Tinctorial stains	
H&E	
PAS	Glycogen (may dissolve out unless alcohol fixation is employed)
Oil red O or Sudan black	Lipid droplets
Gomori's trichrome	Ragged red fibres nemaline rods
Histochemical stains	
Myosin ATP-ase at pH 9.4, 4.6 and 4.3	Fibre type distribution
Oxidative enzymes	
NADH-TR	Myofibrillar architecture mitochondria, target fibres tubular aggregates
Cytochrome c oxidase	Complex IV-negative fibres in many mitochondrial myopathies
Succinic dehydrogenase	Complex II-encoded entirely in nuclear DNA, deficiency is rare
Acid phosphatase	Lysosomes-lipofuschin rimmed vacuoles macrophages
Additional histochemistry for patients with appropriate clinical history suggestive of a metabolic disorder myopathy, e.g. fatiguability, cramps or pains on exercise	
Myophosphorylase	Absent in type V glycogenosis McArdle's disease
Phosphofructokinase	Absent in type VII glycogenosis
Myoadenylate deaminase	MADD deficiency

Fixation for electron microscopy

Electron microscopy is an adjunct to light microscopy in many different diseases, e.g. the mitochondrial cytopathies, nemaline myopathy. Glutaraldehyde gives good preservation of tissue organelles, but because the fixative has only low penetrance only very tiny pieces must be taken. Longitudinal sections are generally the most useful, and very thin strips can be obtained using the dissecting microscope for orientation.

Biochemical and DNA analysis

If it is known beforehand that these investigations are likely to be required and sufficient material is obtained separate pieces can be frozen and stored at –70°C. The reserve tissue from enzyme histochemistry can be used when necessary.

Laboratory procedures

In addition to routine H&E, a limited number of enzyme histochemical techniques and other special stains should be used for preliminary screening of all muscle biopsies. The clinical picture and the initial microscopic findings will dictate the need for further histochemical and immunocytochemical stains.

Immunocytochemistry

The phenotypic spectrum of both dystrophinopathies and limb girdle dystrophies is gradually being broadened. The panel of antibodies relevant to these muscular dystrophies is also continually being increased. Although reduction in any one component of the dystrophin-associated protein complexes often leads to a generalised reduction in all these proteins, this is not inevitable, and it is necessary to use the panel of available antibodies.

A wide range of antibodies that have no diagnostic specificity may, nevertheless, illuminate pathological changes and enhance biopsy interpretation.

Serial sections and levels, longitudinal sections

Serial sections should be used routinely to enable the same fibres to be examined with different stains.

Antibody	Indications
Dystrophin [10] (Antibodies to N and C terminal and to rod portion)	Duchenne and Becker dystrophy Males with an undiagnosed myopathy, mandatory for young males with raised CK Males with cardiomyopathy Women who could possibly be carriers of an Xp21 dystrophy
Adhalin [1, 3] (50-kd dystrophin associated glycoprotein)	Severe childhood autosomal recessive muscular dystrophy (SCARMD) Children with undiagnosed myopathy
Sarcoglycans (α β γ) [1, 3]	Limb girdle syndromes
Laminin [11] (α chain – merosin)	Congenital muscular dystrophy Infants with contractures or undiagnosed myopathy that is not a well-characterised congenital myopathy
Desmin and vimentin	Unclassified congenital myopathies and myopathies with cytoplasmic bodies
Other useful antibodies	
Lymphocyte markers	Biopsies in which mononuclear inflammatory cells are identified.
Ubiquitin, Tau protein [2]	Inclusion body myopathies
N-CAM	Regenerating and denervated fibres
Sialated N-CAM	Denervated fibres
Developmental myosin heavy chain	Regenerating and some denervated fibres
HLA-1	Inflammatory myopathies

Levels are appropriate in a biopsy that is normal or minimally abnormal on preliminary examination, and if an inflammatory myopathy is suspected as inflammation may be patchy.

Transverse sections are preferred for most diagnostic purposes, but longitudinal sections are useful in assessing cores in central core disease, multicore and minicore disease.

Measurements of fibre size and of proportions of different fibre types

Accurate measurement of fibre diameter and assessment of the proportions of different fibre types can be an adjunct to diagnosis. It is not a necessary routine in all biopsies. Significant changes in fibre size and distribution pattern are often obvious from simple inspection, but accurate measurement and quantitation can provide objective evidence. The method of freezing or processing the specimen affects diameter in tissue section; therefore it is important for each laboratory to have constructed its own normal range. A wide variation in normal fibre size, sex and age differences must be recognised. Generalised atrophy may be difficult to appreciate unless compared with the normal range. Various computerised image analysis programmes are available, but most are expensive and frequently cannot achieve separation of adjacent fibres of the same type. Semi-automated systems may be more time consuming but are easy to use and give reproducible results.

Electron microscopy

Electron microscopy is not required for all biopsies but is an adjunct to light microscopy in many different diseases. It is indicated when light microscopy reveals ragged red fibres, usually attributable to aggregates of large abnormal mitochondria, and when light microscopy reveals abnormal inclusions or storage material. It is of particular value in neonatal and infant biopsies, when cytoarchitectural abnormalities are difficult to interpret in tiny fibres.

Preservation of the reserve tissue

The reserve frozen specimen should be stored at –70°C for as long as space permits. It is best stored in a Linde flask of liquid nitrogen, which prevents dehydration and means that if required further frozen sections can be cut successfully months or even years later. The genes responsible for several neuromuscular disorders have been located and cloned in recent years. Others will be added to this list and stored tissue may be used for these analyses. In disorders where the genome has not been cloned stored frozen tissue may be used for RFLP detection and pedigree analysis if another family member presents with a similar condition.

Any paraffin block should be stored permanently or as long as space permits and will be available for future for genetic probing using the polymerase chain reaction.

Table 1 Biopsy interpretation

No abnormalities detected	Does not exclude inflammatory and certain metabolic myopathies	Normal
Atrophy	Small group atrophy	Denervation
	Selective fibre atrophy *alone - not specific but consider*	
	Type 1	Congenital fibre type disproportion, myotonic dystrophy
	Type 2	Many causes *e.g.* connective tissue disease, disuse atrophy, steroid myopathy, systemic illnesses
	Selective fibre atrophy *plus* inflammation	Inflammatory myopathy i.e. polymyositis, dermatomyosistis, etc.
	Perifascicular atrophy	Dermatomyositis, especially juvenile
Fibre type distribution	Type 1 or 2 grouping	Re-innervation
	Type 1 predominance	Congenital myopathy
Necrosis and regeneration	*No* inflammation	Drug induced, metabolic
	Plus inflammation	Inflammatory myopathy
	plus variation in size including hypertrophy and fibrosis	Dystrophy
Inflammation	*Plus* atrophy but rarely hypertrophy *plus* variation in size, including hypertrophy, fibrosis	Inflammatory myopathy e.g. polymyositis, dermatomyosistis Dystrophy
Cytoarchitectural abnormalities *None are disease specific but when numerous consider:*	Inclusions	
	Nemaline rods	Congenital myopathy
	Ragged red fibres – mitochondrial aggregates	mitochondrial myopathy AZT myopathy
	Excess lipid	Metabolic myopathy alcohol, steroids
	Rimmed vacuoles	Inclusion body myopathy
	Excess lysosmes	Acid maltase deficiency juvenile Batten's disease
	Cytoplasmic bodies	Congenital myopathy inclusion body myopathy
	Tubular aggregates	Periodic paralyses myotonia congenita
	Myofibril disarray	
	Targets, core-targets	Denervation
	Cores Lobulate fibres	Central core disease FSH dystrophy, limb girdle dystrophy 2A Bethlem myopathy
	Sarcoplasmic masses Ring fibres	Myotonic dystrophy Myotonic dystrophy,
	if grouped	denervation

Biopsy reporting

Features included in the final report

The biopsy report should include the following features:

Fibre size – presence of atrophy and or hypertrophy, different patterns of atrophy e.g. small group atrophy, perifascicular atrophy, selective fibre type atrophy.

Fibre type distribution – differences from the normal mosaic pattern - type grouping, fibre type predominance.

Cytoarchitectural abnormalities, e.g. central nuclei, target fibres, central cores, ragged red fibres, cytoplasmic inclusions, excess lipid, excess glycogen.

Necrosis and regeneration

Interstitium – cellular infiltration, vasculitis, fibrosis

In addition where appropriate:
i) presence of specific enzymes
ii) presence of immunocytochemical staining for dystrophin, adhalin etc
iii) electron microscopic findings

Diagnostic categories

Morphological changes may be pathognomonic of a specific disease e.g. *nemaline rod body congenital myopathy* (Table 1). However, in many instances, histological abnormalities are not disease specific e.g. *denervation atrophy*, but in the appropriate clinical setting they serve to confirm a suspected diagnosis (Table 1). If a specific diagnosis is not possible the report should identify a diagnostic ostic category.

1. Normal
2. Denervation
3. Inflammatory
4. Type 2 fibre-selective atrophy (not disease specific)
5. Type 1 fibre-selective atrophy (not disease specific)
6. Dystrophic
7. Congenital myopathy
8. Metabolic disorder – glycogenosis, lipid storage, mitochondrial
9. Vasculitic, ischaemic
10. Unspecified – abnormalities present, but not diagnostic

Further action

The final report should indicate whether further investigations are indicated, e.g.

Biochemical enzyme assays: for mitochondrial enzymes
for alpha-1-glucosidase

Genetic studies: for mutations in the dystrophin genome
for mutations in mitochondrial DNA

Specialised biopsies

Motor end-point biopsy

Motor end-point biopsy, which aims to include motor end-plates in the biopsy, is a specialised technique which has a role in the diagnosis of the myasthenic syndromes. In these disorders methylene blue vital staining or silver impregnation shows fine terminal sprouts or elongation of the end-plates, but electron microscopy is required to identify diagnostic abnormalities of end-plate morphology and distribution of acetyl choline receptors as shown by labelling with alpha bungarotoxin. The motor end-point band must be located and marked on the skin by external electrical stimulation and then discrete location obtained by stimulation of the exposed muscle. Motor end-point biopsy should only be performed in conjunction with appropriate detailed electrophysiological studies.

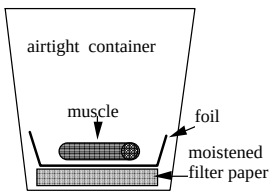
Biopsy for suspected malignant hyperpyrexia

Malignant hyperpyrexia is due an abnormal susceptibility to certain inhalational anaesthetic agents and muscle relaxants that trigger sustained muscle contraction and hyperthermia. The abnormal response is occasionally seen in patients with a recognised myopathy, particularly central core disease, but others with an autosomal dominant trait may have no overt physical disability, and hence the first indication is an adverse reaction to anaesthesia. Following such an incident, muscle biopsy is indicated, but should only be performed in a specialised centre where in vitro testing is available. Any histological abnormalities are usually minor and non-diagnostic.

Appendix A

Transport of the muscle biopsy specimen

Container



Transport medium

This medium can be used for the transportation of fresh muscle. The results are far less satisfactory than with snap frozen muscle and it is not suitable for all enzyme histochemical preparations.

Buffer

0.025 M potassium citrate

(0.81 g in 100 ml distilled water MW = 324.41)

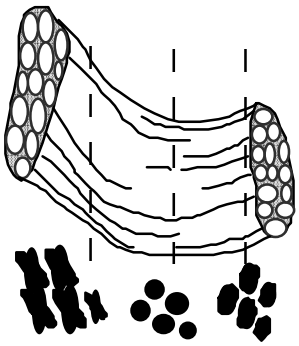
0.005 M magnesium sulphate

(0.0692 g in 100 ml distilled water MW = 138.38

Fixative

Mix 100 ml of buffer (as above) plus 55 gm ammonium sulphate. Check pH 7.0 and adjust with 1 M KOH

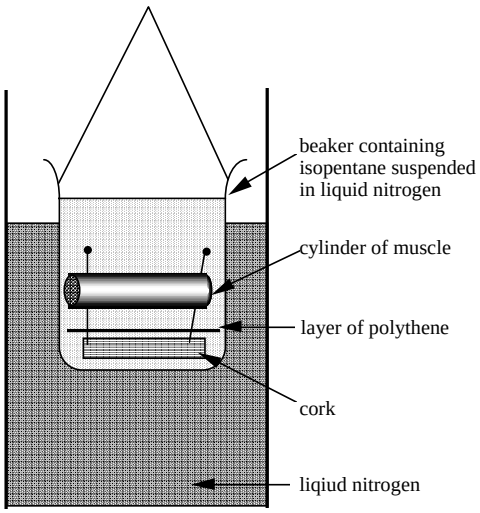
Specimen orientation



Elongated profiles are obtained in oblique sections. True transverse sections are required for accurate measurement of fibre diameter and comparison of fibre size.

Freezing the specimen

The conventional method of freezing tissues by direct immersion in liquid nitrogen is unsuitable for muscle. Liquid nitrogen cools the muscle too slowly and it is subject to ice crystal artefact, which hinders interpretation of pathology. The following method eliminates the artefact by ensuring almost instant – "snap" – freezing of



the specimen. One the specimen is frozen it is essential that it is handled only with cooled instruments. Gripping the specimen with forceps at room temperature will cause partial thawing which again induces artefact. For long term storage immersion in a Linde flask of liquid nitrogen is more satisfactory than a freezer, as the specimen tends to dehydrate in the latter.

A small cylinder of skeletal muscle (up to 1.0 cm long × 0.3 cm diameter) is orientated correctly with the fascicles running longitudinally. It is placed on a small strip of polythene and then fixed to a piece of cork by means of a fine (i.m. injection type) needle inserted at each end. Slight tension may be applied, but the specimen should not be stretched. This fixation prevents hypercontraction of the specimen. The specimen is gently eased up off the polythene so that it is strung between the two needles like a clothes line.

The polythene prevents the muscle from sticking to the cork, and the specimen should not be in contact with anything other than the needles before it is frozen. A small glass beaker filled with liquid isopentane is lowered into liquid nitrogen as shown in the diagram. The two liquids are *not* miscible. Initially the liquid nitrogen around the beaker will vaporise but as the isopentane cools the vapour clears and the operator will see white solid beads appearing on the bottom of the beaker. The isopentane is then at the correct temperature. The specimen mounted on cork is dropped in and it will freeze immediately, becoming rigid. It should be lifted out with forceps that have also been cooled in liquid nitrogen, snapped off the fine needles and placed in a suitable cooled container (e.g. a small plastic tube) or transferred to the cryostat. The operator will find it easier to wear cotton gloves to handle the cold instruments.

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