Muscle Biopsy

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ABSTRACT
Muscle biopsy has been used for a long time for diagnosis of muscular, neurogenic and systemic disorders with muscle involvement, because only very few of these disorders show sufficient specific clinical features for definite diagnosis. Since the presence of difficulties in the screening of numerous genes, muscle biopsy could be a time and cost effective procedure for solving the diagnostic problems. The aim of this article is to mention the importance of muscle tissue in the evaluation of primary and secondary muscle diseases, special consideration of how to biopsy, handling the specimen and performing the special staining, and the microscopic findings in order to have better interpretation results.

Keywords: Muscle, Biopsy, Pathology

Introduction

The removal of a small piece (10-15mg) of muscle tissue for examination is called “muscle biopsy” (1). It is usually performed when the patient is awake and feels little or no discomfort. There are two essential ways for biopsy:

1-Needle biopsy is using a needle which inserts into muscle for taking the specimen, it is less time-consuming, more cost-effective with fewer complications than an open biopsy but obtaining a small and often traumatized sample (2,3) so could be preserved when peripheral nerve sampling is not required and when large tissue samples are not needed for extensive biochemical analyses (4).

2-Open biopsy is performed after a small cut in the skin to take samples and is preferred because the physician (surgeon, pathologist or neurologist) is able to inspect the tissue (especially important in patchy involvements of muscle) and then take the sample. Studying the muscle tissues is a golden tool in diagnosis of muscle disease, some of the primary neurogenic disorders, some of the systemic diseases with muscle involvement and also differentiation and categorization of the primary muscle disorders (5).

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General Considerations
First of all, the physician or the teamwork should be aware about the indications and contraindications of biopsy.

Indications
1- Evidences of muscle disease: weakness and discomfort, muscle cramps, post exercise early fatiguibility (non-myasthenic)
2- Increase in SCK (Serum Certain Kinase) 
3- Myopathic pattern in electromyography (EMG)
4- Systemic disease which could have muscular involvements

Contraindications
1- The diseases which could be diagnosed by electric diagnostic tools such as myasthenia gravis (MG), myotonia and CMS (congenital myasthenic syndrome) and also could be confirmed by mutation detection methods.
2- Traumatized or injected muscle or the muscle which has undergone electromyography till minimally one month after trauma or the procedure.
3- Muscle with severe weakness (which may show severe necrotic features in biopsy)

Muscle of Choice
The muscle of choice for biopsy is characterized by physical exam or imaging techniques such as Magnetic Resonance Imaging (MRI). MRI also could reveal the pattern of muscle involvement and facilitates the differential diagnosis.
The best muscle for biopsy is a moderately involved muscle. Deltoid, biceps and quadriceps muscle are preferred, however in special instances, special muscles such as, orbicularis oculi is recommended for the diagnosis of mitochondrial myopathies, using samples which are taken during belfaroplasty of ptosis surgery in order to reduce morbidity and cost.

Muscle biopsy preparations
Many of the muscle morphological abnormalities such as regeneration, fibrosis, fatty infiltration and vaculated fibers are evident in hematoxylin & eosin stained sections, but none of the muscle disorders especially muscular dystrophies could be distinguished on the basis of muscle histology.
For biopsies which are intended to investigate inflammatory diseases (dermatomyositis, polymyositis, inflammatory myopathy) the tissue should be divided to frozen for enzyme histochemistry, formalin-fixed for hematoxylin & eosin (H&E) staining, and
glutaradheyde- fixed for electron microscopy (EM).

For investigation of muscular dystrophies (Duchenne Muscular Dystrophy (DMD), Becker Muscular Dystrophy (BMD), Limb-Girdle Muscular Dystrophy (LGMD), Fascio-scapulo-Humeral Dystrophy (FSHD) and Myotonic Dystrophy (MD) the tissue should be divided to formalin fixed for H&E, frozen for enzyme histochemistry & immunohistochemistry.

If the biopsy is intended for evaluation of a metabolic abnormality of mitochondrial myopathy (MELAS, Kearn-Sayre syndrome Lipid storage disease) the tissue should be divided to frozen for enzyme histochemistry, and histochemistry and glutaraldehyde-fixed for EM.

Specimen preparation

**Fresh tissue:**
1. The muscle sample should be divided minimally to three parts (as will be described below) and transferred to the laboratory within 30 minutes.
2. The 1st sample (only 2mm in diameter) is clamped and is fixed in glutaraldyhyde for electron microscopy. The 2nd sample, for histochemistry and light microscopy is oriented longitudinally and placed on saline-moistened paper or gauze for transferring to the pathology laboratory where it is frozen or fixed in formalin. The 3rd sample is also placed on saline-soaked paper and reserved for biochemical studies in the cases they may be indicated (15).

**Frozen Tissue:**
The best results for enzyme histochemistry & immunohistochemistry studies are obtained when tissues are frozen rapidly and kept in -80ºC until sectioned. Any thawing and re-friezing leads to ice crystal formation with loss of morphologic details and cell membrane integrity (surface antigens). Enzymatic activity can also be lost during thaw& freeze (16).

**Freezing Methods**
1. Liquid nitrogen: tissue for biochemical studies.
2. Isopentane /liquid nitrogen: tissue for enzyme histochemistry and immuno-histochemistry studies.

Residual tissue, with preserved orientation, may be immersed in formalin for routine histology. 10% natural buffered formalin (NBF) is suitable with minimum 4 hours fixation time. Muscle may be fixed while still clamped.

**Findings**
After studying the muscle, the pathologist could be able to answer the following issues (17, 18):
- Size, shape, type of muscle fibers, internal architectures and storage materials.
- Myopathic or neurogenic pattern of involvement (round: myopathic & angular: neurogenic).
- Distribution of atrophic fibers (if present): grouped or scattered.
- Fiber type smallness:
  - Small type1: Hereditary myopathy
  - Small type 2: Congenital myasthenia gravis
- Acute or chronic inflammatory process (if seen):
  - Acute: -myopathy: muscle fiber degeneration & regeneration
  - Neuropathy: Small angular fibers
  - Chronic: -myopathy: fiber hypertrophy & increase in endomysial connective tissue
  - Neuropathy: fiber type grouping, pyknotic nuclear chromatin

Abnormalities in :
- Vessels
- Connective tissue: endo & perimysial
- Intramuscular nerves

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Histochemistry stains
The golden data could be extracted from different methods of staining; each could be applied for a special goal or to differentiate a few disorders which could not be clinically differentiated or categorized. The most common histochemistry stains are as the Table 1(17).

**Table 1- Muscle biopsy histochemical stains**

<table>
<thead>
<tr>
<th>Category</th>
<th>Method</th>
<th>Utility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td>Hematoxylin and eosin</td>
<td>Muscle fiber pathology; Nuclei</td>
</tr>
<tr>
<td></td>
<td>Verhof van Giesson (VvG)</td>
<td>Connective tissue; vessel structure intramuscular nerve</td>
</tr>
<tr>
<td></td>
<td>Gomori Trichrome</td>
<td>Connective tissue; Nemaline rods</td>
</tr>
<tr>
<td>Fiber Type Enzyme</td>
<td>Myofibrillar ATPase</td>
<td>Muscle fiber type grouping or atrophy</td>
</tr>
<tr>
<td></td>
<td>ATPase pH 9.4</td>
<td>Myosin loss; type 1 or 2 fiber atrophy</td>
</tr>
<tr>
<td></td>
<td>ATPase pH 4.6</td>
<td>Type 2B muscle fibers</td>
</tr>
<tr>
<td></td>
<td>ATPase pH 4.3</td>
<td>Type 2C (Immature)</td>
</tr>
<tr>
<td></td>
<td>NADH-TR</td>
<td>Muscle fiber internal architecture; tubular aggregates; cores</td>
</tr>
<tr>
<td>Oxidative Enzymes</td>
<td>Succinate dehydrogenase</td>
<td>Mitochondrial pathology</td>
</tr>
<tr>
<td></td>
<td>Cytochrome oxidase</td>
<td>Mitochondrial pathology</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mitochondrial DNA encoded protein</td>
</tr>
<tr>
<td>Glycolytic Enzymes</td>
<td>Phosphorylase</td>
<td>Phosphorylase deficiency</td>
</tr>
<tr>
<td></td>
<td>Phosphofructokinase (PFK)</td>
<td>PFK deficiency</td>
</tr>
<tr>
<td></td>
<td>Acid phosphatase</td>
<td>Macrophages; Lysosomes; Lipofuscin</td>
</tr>
<tr>
<td></td>
<td>Non-specific esterase</td>
<td>Macrophages; lysosomes;</td>
</tr>
<tr>
<td>Hydrolytic Enzymes</td>
<td>Acetylcholinesterase</td>
<td>Neuromuscular &amp; myotendinous junctions</td>
</tr>
<tr>
<td></td>
<td>Alkaline phosphatase</td>
<td>Denervated (small angular) muscle fibers</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Neuromuscular &amp; myotendinous junctions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Regenerating muscle fibers; Immune disease of connective tissue</td>
</tr>
<tr>
<td></td>
<td>PAS</td>
<td>Glycogen &amp; Carbohydrate disorders</td>
</tr>
<tr>
<td></td>
<td>Alcian blue</td>
<td>Mucopolysaccharide</td>
</tr>
<tr>
<td>Storage material</td>
<td>Sudan black B</td>
<td>Lipid storage</td>
</tr>
<tr>
<td></td>
<td>Oil red O</td>
<td>Lipid storage</td>
</tr>
<tr>
<td></td>
<td>Congo red</td>
<td>Amyloid; Inflammation; Vacuoles</td>
</tr>
<tr>
<td></td>
<td>Myoadenylate deaminase</td>
<td>AMPDA deficiency</td>
</tr>
<tr>
<td></td>
<td>Methyl green pyronine</td>
<td>RNA</td>
</tr>
<tr>
<td>Other</td>
<td>Acridine orange</td>
<td>RNA</td>
</tr>
<tr>
<td></td>
<td>Von Kossa</td>
<td>Calcium</td>
</tr>
<tr>
<td></td>
<td>Alizarin red</td>
<td>Calcium</td>
</tr>
<tr>
<td>Fixed muscle</td>
<td>Toluidine blue</td>
<td>Muscle fibers; Capillaries</td>
</tr>
</tbody>
</table>
Protein Analysis
Histological and histochemical analysis should be completed with protein analysis. Protein analysis could be assessed through immunohistochemistry and western blot analysis (19).

Immunohistochemistry
There are some immune stains which are very important in differentiation of some hereditary muscle disorders such as muscular dystrophies. For example Dystrophin analysis by immunohistochemistry is a very sensitive method for diagnosis of DMD and BMD (20, 21).

The immune stains reveal expressed or absent protein in the muscle tissue as the result of one or more mutations in the corresponding genes. This process is a golden method which the pathologist could follow & confirm the mutant gene products and could be performed with fluorochrome (immunofluorescence) or peroxidase (22).

In some instances, secondary protein changes (e.g. Utrophin expression) in the absence of another type of protein (e.g. Dystrophin) as we see severely in DMD and mildly in most cases of BMD could be assayed using immunohistochemistry in two different tissue sections (23). Utrophin is a dystrophin homologue which is upregulated in the absence or reduction of dystrophin; it should be also noted that the intensity of utrophin expression isn’t related to disease severity (24). Symptomatic mother carriers of mutated dystrophin gene (either with clinical symptoms and/or increased SCK levels) could be examined by utrophin which may result in on/off expression pattern of protein (25-28). Reciprocal expression between dystrophin & utrophin is also evident in these cases (28).

Table 2 shows some of the immunostains and their potential diagnostic roles.

Table 2- Commonly used antibodies as immunostains in protein analysis method

<table>
<thead>
<tr>
<th>Disease</th>
<th>Antibody</th>
<th>Protein-based diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dystrophinopathy (DMD and BMD)</td>
<td>Dystrophin</td>
<td>Immunohistochemistry, Western blot analysis</td>
</tr>
<tr>
<td>Calpainopathy LGMD-2A</td>
<td>Calpain</td>
<td>Western Blot analysis not for immunohistochemistry</td>
</tr>
<tr>
<td>Dysferlinopathy LGMD-2B</td>
<td>Dysferlin</td>
<td>Immunohistochemistry, Western blot analysis</td>
</tr>
<tr>
<td>Sarcoglycanopathy LGMD2C-F</td>
<td>Sarcoglycan</td>
<td>Immunohistochemistry, Western blot analysis</td>
</tr>
<tr>
<td>Telethoinopathy LGMD-2G</td>
<td>Telethoin</td>
<td>Immunohistochemistry, Western blot analysis</td>
</tr>
<tr>
<td>Congenital muscular dystrophy</td>
<td>Merosin (Laminin α2)</td>
<td>Immunohistochemistry, Western blot analysis</td>
</tr>
</tbody>
</table>
Immunoblotting
The quality and quantity of the target protein could also be assessed by western blot analysis using muscle extract. This procedure could offer the clues of the mutation type (11, 28, 29). Immunoblotting is a suitable method for detecting the missed or reduced protein in muscle tissue, and could be performed as multiplex western blot analysis (30, 31). This procedure is preferred by some centers instead of tissue immunostaining in all affected males suspected for DMD (1).

Genetic studies
As mentioned above, muscle biopsy could provide the material for immunoblotting in order to detect abnormal or absent proteins, however mutation detection techniques are also available using blood samples (32). If the mutation could not be detected with general methods, another techniques such as Single-Strand-Conformation-Polymorphism (SSCP), Polymerase Chain Reaction (PCR) and Southern blot analysis are recommended (1).

Conclusion
The precise study of muscle tissue is a valuable method for detecting muscle disorders, primary and also secondary, if it follows standard protocols. Routine histology could not be used for diagnosis of muscular dystrophies and in most instances methods of protein analysis recommended.

Acknowledgements
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References