Skeletal Muscle Pathology in Mitochondrial Diseases

Endre Pál*
Department of Neurology and Neuropathology Unit, Department of Pathology, University of Pécs, Hungary

*Corresponding author: Endre Pál, Department of Neurology, University of Pécs, Pécs, Rét u. 2, Hungary, Tel: +36 72 535 900; Fax: +36 72 535 911; Email: pal.endre@pte.hu

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ABBREVIATIONS


INTRODUCTION

Mitochondrial diseases (MD) are one of the most frequent among neuromuscular disorders. The estimated frequency is between 9.2/100.000 and 20/100.000 worldwide [1,2].

The clinical diagnosis of the classical mitochondrial syndromes such as MELAS (Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes syndrome), MERRF
(Myoclonic epilepsy and ragged red fibers), KSS (Kearns-Sayre syndrome), MNGIE (Mitochondrial neuro-gastrointestinal encephalopathy), etc. sometimes is difficult, because the phenotypes are often incomplete, and so-called non-syndromic forms frequently occur, where wild spectrum of symptoms resembling peripheral and central nervous system appears.

The diagnosis of MDs usually based on diagnostic criteria. These diagnostic criteria includes clinical data (e.g. typical or atypical multisystem mitochondrial syndromes), biochemical alterations (e.g. decreased respiratory chain (RC) enzyme activity, impaired ATP synthesis, lactate acidosis), histological features (skeletal muscle mitochondrial alterations and brain pathology) and genetic results (mutation of mitochondrial DNA (mtDNA), or nuclear mitochondrial genes) [3-5].

The diagnostic procedure includes detailed neurological, ophthalmological, audiologic, cardiologic and internal medicine exams. The following diagnostic tests can be performed: Easy, non-invasive test is the determination of resting and exercise-induced raise of lactate. Cycle-test was judged as unspecific and can be negative in certain cases (especially in patients with mild myopathy, LHON (Leber hereditary optic neuropathy) and PEO (Progressive external ophthalmoplegia) [6]. The handgrip test was reported to be more sensitive [6,7]. Serum creatin kinase (CK) is again unspecific marker, might be normal, or slightly elevated. Electromyography (EMG) might prove myogenic changes in the involved muscles, but it is frequently normal. Electroneuronography (ENG) is able to demonstrate axonal neuropathy in certain cases. Muscle imaging usually shows non-specific atrophy of limb muscles. 31phosphorus magnetic resonance spectroscopy (MRS) is a specific, but not a sensitive tool for demonstration of decreased phosphocreatine amount in skeletal muscles [8]. Muscle biopsy is an invasive, but usually unavoidable procedure in majority of cases with suspected mitochondrial diseases. Beyond the characteristic morphological features, biopsy sample is suitable for biochemical tests (measurement of RC enzyme activity) [9] and genetic tests [10].

**SKELETAL MUSCLE BIOPSY**

In the last decades two types of biopsy procedure were developed: the open and the needle biopsy. The advantage of open biopsy is that we can acquire large enough piece of tissue suitable for morphological analysis. Needle biopsy is less invasive and sufficient for molecular and biochemical tests.

**Indications and Contraindications**

**Indications:**

- Evidence of muscle disease
- Weakness with characteristic pattern, permanent muscle pain, cramps, fatigability
- Significantly raised blood creatine kinase level
• Myogenic alterations on EMG
• Mitochondrial syndrome with skeletal muscle involvement
• If neurogenic muscular atrophy is suspected muscle biopsy is not routinely indicated, but in cases where genetics (e.g. spinal muscular atrophy), or clinical investigations (e.g. amyotrophic lateralsclerosis) are doubtful
• To prove muscular involvement in systemic disorder
• Sarcoidosis, vasculitis, amyloidosis

There are no major contraindications for biopsy except anticoagulant usage.

**Muscle Biopsy Procedure**

Actually the most popular method is the “semi-open biopsy” using of the Weil-Blakesley conchotome introduced by Henrikkson in 1979 [11]. The procedure is simple and fast, allows removing a quite big sample, and the frequency of complications is low [12]. The most common places of biopsy are deltoid, biceps, quadriceps and anterior tibial muscles.

**Sample Handling**

The removed tissue may be transported in humid chamber for few hours. One piece should be frozen in isopentane precooled in liquid nitrogen for light microscopic evaluation. A second piece is fixed in glutaraldehyde, and embedded in plastic for ultrastructural evaluation. A third piece is stored in liquid nitrogen for molecular studies.

**Light Microscopic (LM) Procedures**

For light microscopy a standard battery of stainings is usually performed as recommended [13]. Hematoxylin and eosin (HE) staining shows the fascicular structure, the size and shape of fibers, location of nuclei and various forms of muscle fiber damages can be seen. Modified Gomori trichrome (mGT) highlights the connective tissue accumulation and helpful to detect mitochondrial accumulation (ragged red fibers, RRF), rods and vacuoles. Periodic acid-Schiff (PAS) demonstrates glycogen; oil red or sudan black staining shows lipid content. ATP-aze enzyme histochemistry allows to evaluate the fiber type distribution. Nicotinamide adenine dinucleotide hydrogen tetrazonium reductase (NADH) enzyme is located both in mitochondria and in sarcoplasm, therefore it shows the internal architecture of fibers and its abnormalities (e.g. cores, target fibers, tubular aggregates). NADH, succinic dehydrogenase (SDH), cytochrome c oxidase (COX) enzyme histochemistry is suitable for demonstration of mitochondrial content and function (such as accumulation of mitochondria, activity of RC enzyme complexes: I (NADH), II (SDH) and IV (COX)). Acidic phosphatase histochemistry highlights lysosomal activity and macrophages.
Electron Microscopic (EM) Evaluation

The plastic embedded sample is proceeded for EM study. It is helpful in cases with suspected metabolic myopathies to prove the accumulation of certain substances (glycogen, lipids), in mitochondrial diseases to demonstrate mitochondrial pathology, in congenital myopathies to find structural alterations and demonstrate inclusions.

Molecular Tests

The frozen tissue can be stored in liquid nitrogen for long time, and it is suitable for protein tests (e.g. for immunoblotting), biochemical tests (measurement of RC chain enzyme activity, or other enzymes involved in glycolysis), messenger RNA studies (e.g. fluorescent in situ hybridization (FISH), reverse-transcriptase polymerase chain reaction (RT-PCR)) and DNA tests, as well.

SKELETAL MUSCLE PATHOLOGY IN MITOCHONDRIAL DISEASES

The Source of Muscle Sample

In suspected mitochondrial cases the previously mentioned muscles are chosen for biopsy. Proximal muscles are preferred, the dystrophic muscles usually are avoided. Extraocular muscles are frequently involved and pathology of those muscles are more prominent, but biopsy is not common, just in cases during eyelid surgery for correction of ptosis [14].

Light Microscopic Alterations in Mitochondrial Diseases

Wild spectrum of mitochondrial pathology can be found is skeletal muscle ranging from mild, non-specific myopathic changes to severe mitochondrial damage. The pathology depends on the patient’s age, the type of clinical phenotype and the duration of the disease.

Accumulation of mitochondria

Accumulation of mitochondria predominantly in the subsarcolemmal space develops non-specifically in variety of diseases including metabolic, endocrine, inflammatory myopathies and muscular dystrophies, as well [15,16]. Therefore the diagnostic value of isolated accumulation of mitochondria is low. In MDs the subsarcolemmal accumulation of mitochondria usually is prominent, it is best visualized by the mGT method: mitochondria appear as a red rim at the periphery of muscle fibers (Figure 1).
Figure 1: Grading of mitochondrial accumulation. A and B: mild, C: moderate, D: marked, diffuse accumulation of mitochondria in the subsarcolemmal space. mGT, original magnification: 160x.

Ragged red fibers

During LM evaluation the pathological hallmark of MDs is the presence of ragged red fibers (RRF). This ragged appearance of fibers sometimes can already be seen in HE specimen (Figure 2), but these fibers are typically recognized with mGT staining with a red peripheral rim, or with granular red appearance of the sarcoplasm. The red staining is the result of the increased amount of phospholipids in the membranes of proliferated mitochondria. Figure 2 demonstrates some examples of RRFs. RRF develops due to the proliferation of abnormal mitochondria and contains a high level (85-90%) of deleted, or mutated mtDNA [17-19]. It is important to note that RRFs are common in certain MDs, such as PEO (regardless of type of mutation), KSS, MNGIE (with single or multiple mtDNA deletion), but rare, or absent in LHON and Leigh syndrome [20]. The other important point is the age of the patients. RRFs are rarely seen in infantile MDs, more common in adult, or old MD patients, and can be found during normal aging [21-23]. The frequency of RRFs may vary among wide limits. According to the diagnostic criteria established by Bernier and Taylor, MDs is suspected if the amount of RRFs >5% above age of 50 years, or >2% between age 30-50. If the patient is younger than 30 years, any RRFs judged to be pathologic [3-5].
Figure 2: Ragged red fibers. Granular appearance of RRF can be seen on HE (A), but clearly visible with mGT staining (D). Various types of RRFs on mGT from MD cases (B,C,E,F). Original magnification: A,C, D and E: 160x, B and F: 200x.

There are some technical concerns regarding to RRFs. First is the handling of the sample. Inadequate manipulation, pressure by the surgeon can cause abnormal increased staining on mGT that has to differentiate from pathologic changes. Second, only fresh frozen tissue is suitable for adequate mGT procedure, because either postmortem delay or fixation results in artificial staining.

Again, RRFs might be commonly present in other neuromuscular diseases (metabolic myopathies, dystrophies, inflammatory myopathies, especially inclusion body myositis (IBM)) [22].

RRFs frequently show moderate glycogen and lipid accumulation with PAS and oil red staining, respectively. Marked lipid accumulation in muscle fibers occurs in CoQ10 deficiency [24].

Ragged blue fibers

Accumulated mitochondria in the subsarcolemmal region are usually visible more clearly with oxidative enzyme reactions, such as NADH, SDH and COX. Another, even more sensitive example of mitochondrial proliferation is the so called “ragged blue” appearance of fibers with SDH enzyme histochemistry (Figure 3). SDH enzyme histochemistry represents the activity of complex II in RC. The ratio of ragged blue fibers usually outnumbers the amount of RRFs visible in mGT samples.


**Figure 3:** Ragged blue fibers in a biopsy sample from a MELAS case. Several, scattered ragged blue fibers showing increased SDH enzyme activity. Original magnification: 80x.

**Cytochrome-c oxidase (COX) negative fibers**

COX activity represents the function of complex IV in RC. The COX activity more easily can be evaluated on slides submitted a sequential SDH-COX double enzyme histochemistry, because this technique results better contrast and SDH usually is preserved. Two forms of decreased COX activity can be observed: (A) diffuse decrease, of absence of activity in cases of infantile COX deficient myopathy [25] and Leigh's syndrome due to mutation of genes coding assembly proteins (e.g. SURF-1) [26], or (B) scattered COX deficient fibers. The latter can be associated with RRFs (RRF without COX activity) or COX deficient fibers may exist in muscles devoid any RRFs. It is usual that the number of COX negative fibers higher than RRFs (Figure 4 and Figure 5).

**Figure 4:** COX activity of ragged blue fibers. RRFs on mGT (A and D) are clearly visible on SDH staining as ragged blue fibers (B and E). Ragged fibers can show increased (C) or decreased (F) COX activity. MELAS cases. Original magnification: A,B,C: 200x, D,EF: 80x.
Scattered COX negative fibers, or COX negative RRFs represent the most common pattern observed in muscle biopsies; it is not specific, occurs in the majority of MDs, more commonly in PEO, KSS, and MERRF. In opposite, COX positive RRFs characteristically develop in MDs due to structural gene defects except COX genes. Accumulation of deleted mtDNA was demonstrated in COX deficient fibers regardless their ragged or non-ragged appearance in mitochondrial myopathy. There was no correlation between the proportion of COX deficient muscle fibers and the amount of deleted mtDNA [25,27]. Similarly, high proportion (>85%) of the A3243G point mutation was confirmed in COX negative fibers in MELAS [17,18,28].

We frequently find COX negative fibers in acquired myopathies (IBM, dermatomyositis, statin myopathy, endocrine myopathies). Characteristically, in certain forms of dermatomyositis COX negative fibers are located in the perifascicular region, this condition implicates worse prognosis [29].

Technical note: careful evaluation of serial sections, or different parts of the samples is recommended because of the segmental nature of COX enzyme deficiency. The accumulation of the mitochondria containing mutated mtDNA sometimes is not continuous, instead focal, therefore the absence of COX activity involves one or more segments of a muscle fiber such as demonstrated in Figure 6 (30,31). Matsuoka et al. reported correlation of the length of segmental loss of COX activity with the proportion of deleted mtDNA (31).
Figure 6: Segmental loss of COX activity. Longitudinal section of a muscle fiber showing segmental loss of COX activity (blue) while in the other segment the COX activity is preserved (brown, COX-SDH enzyme histochemistry, 160x).

Pathology of vessels

MELAS is characterized by various functional problems of the central nervous system, including stroke-like episodes in young age and migraine. A specific morphological feature of MELAS that might be related to vascular events, is the strong SDH reactivity of the walls of blood vessels (strong SDH reactive vessels, SSVs) seen in muscle biopsy specimen (Figure 7) [28,32], caused by the accumulation of mutated mitochondria. Microdissected blood vessels from MELAS patients showed a high percentage of mutant genomes that was comparable with the amount found in RRFs in same patient [28,33]. Although SSVs are specific for MELAS, but the frequency might be quiet low in muscle biopsy sample [34].

Figure 7: Strong SDH-reactive blood vessel in a muscle biopsy specimen from a MELAS patient (SDH histochemistry, 200x).
Immunohistochemistry

Proteins of RC complexes are encoded by mtDNA and nuclear DNA (nDNA), except proteins of complex II, which are coded by only nDNA. Recently, a wide range of antibodies became commercially available against the subunits of RC complexes.

Anti-COX subunit antibodies can be useful to detect the site of protein/genetic defect (nuclear versus mitochondrial) by comparing the nuclear encoded (COX IV) to mtDNA encoded COX subunits (COX I, II, and III) in a single fiber level.

Moraes et al. found correlation between COX enzyme activity and reduced staining with antibodies against mtDNA encoded COX II subunit, while staining of the nuclear encoded protein (COX IV) was preserved [28].

In muscle fibers with absent or decreased COX enzyme activity the antibody pattern is variable, might help to differentiate fatal and benign type of COX deficiency [35] and might drive the further biochemical and genetic investigations [36,37].

Electron Microscopic (EM) Findings in Mitochondrial Diseases

Ultrastructural investigation demonstrates the accumulation of normal or damaged mitochondria. However, because of the lack of specificity of mitochondrial alterations, EM evaluation has low priority in the diagnostic procedure of MDs.

We can find mitochondrial accumulation among muscle fibrils and more prominently in the subsarcolemmal region. Several forms of mitochondrial alterations (enlarged, elongated, ring-shaped, bizarre-shaped mitochondria; concentric, or thickened cristae, paracrystalline inclusions) are typical feature of MDs, but can be detected almost in all types of myopathies [38,39].

Mitochondrial alterations are commonly followed by accumulation of glycogen and lipid vacuoles. In advanced cases, variable degree of myofibril loss develop, as well (Figure 8).
**Figure 8:** Electron microscopic demonstration of mitochondrial alterations. Accumulation of mitochondria in the subsarcolemmal (A) and in the interfibrillar space (B,C). Accumulation of bizarre-shaped mitochondria and lipid vacuoles in the interfibrillar space (C). Collection of abnormal mitochondria: enlarged mitochondrion, the internal structure is replaced by glycogen and elongated mitochondria with paracrystalline inclusions (D), mitochondrion with abnormal, concentric cristae (E). Mitochondrion with destructed internal structure replaced by needle-shaped inclusions (F), elongated mitochondrion with paracrystalline inclusions and mitochondria with destruction of cristae (G). Magnification: A: 2500x, B and C: 12000x, D: 15000x, E and G: 30000x, F: 40000x.

**Biochemical Analysis of Muscle Sample**

Histopathological studies may be supported by quantitative biochemical assay of RC enzymes.

Fresh or frozen muscle samples are suitable for enzymology. Importantly, RC enzyme activity must be normalized to a control enzyme, usually citrate synthase. Careful sample handling, experienced laboratory staff and standardized controls are necessary for correct assessment. Individual or combined loss of activity of RC enzyme complexes can be found and the results might drive the genetic analysis [40-42].

**MITOCHONDRIAL SYNDROMES**

**Mitochondrial Myopathy**

One of the most common clinical phenotype of MD is the isolated myopathy. However, myopathy is frequently associated with other peripheral and central nervous system involvement. The pathological picture is variable depending of the type of genetic defect. If COX enzyme complex is
involved (e.g. mtDNA deletion, COX-I mutation) RRFs and COX negative fibers are present [43]. Similarly, several RRFs and numerous COX negative fibers (40-50%) occur in mtDNA encoded tRNA mutation diseases [37]. If the COX complex devoid of biochemical defect (e.g. point mutation in ND genes, or cytb gene) COX positive RRFs are present in muscle samples [44].

PEO, KSS, MNGIE

Although the genetic background of these disorders is heterogeneous, the pathological findings in skeletal muscle biopsy are similar.

KSS is usually sporadic disease caused by the single mtDNA mutation. The clinical picture is characteristic: onset before the age of 20 years, low stature, external ophthalmoplegia, retinitis pigmentosa, sensorineural deafness, atrioventricular block and cerebellar signs. Muscle biopsy contains high amount of COX negative RRFs.

The majority of sporadic PEO cases harbor single mtDNA deletion or mtDNA point mutation. In these cases muscle pathology reveals many RRFs and COX negative fibers [45]. Laforet et al. reported a large series of patients with PEO due to mtDNA deletion or point mutation. Majority of patients had RRFs. The proportion of COX negative fibers ranged between 1-35%, but no significant relationships were found among COX deficiency, ratio of RRFs and genetic alterations [45]. Goto et al. found positive correlation between the number of COX deficient fibers and the proportion of deleted mtDNA [46].

In recessive PEO cases due to POLG1 mutation the histological findings are variable. The muscle histology can be normal, especially in pediatric cases. COX negative RRFs were reported in myopathy cases, but RRFs with increased COX activity was found in SANDO (sensory ataxic neuropathy, dysarthria and ophthalmoparesis) patients [47,48].

Muscle biopsy in dominant PEO patients due to PEO1 (Twinkle) gene mutation showed mitochondrial abnormality with variable severity including single to numerous COX negative fibers [49].

RRM2B-associated PEO syndromes usually characterized by multisystem involvement beyond ophthalmoparesis. The muscle histopathology is not unique, RRFs and COX negative fibers occur, more commonly in recessive than dominant forms [50].

MNGIE is caused by the mutation of thymidine phosphorylase gene which results in high serum thymidine level. The clinical alterations include gastrointestinal dysmotility and cachexia, external ophthalmoplegia, sensorimotor neuropathy and (subclinical) leukoencephalopathy. Skeletal muscle biopsy shows RRFs and COX negative fibers, although negative biopsy findings were reported, as well [51].
Diseases Due to Heteroplasmic mtDNA Mutations

MELAS

Skeletal muscle biopsy might be normal in MELAS [28]. The dominant finding is the presence of COX positive RRFs, but COX negative RRFs might occur [33,52]. Immunohistochemistry shows the reduction of mtDNA encoded proteins (COX-II, ND1), while the amount of nDNA encoded proteins is normal [28]. Vascular pathology (SSVs) is a specific alteration, but its occurrence is variable, sometimes as low as 14% [34].

MERRF

MERRF is a maternally heritable MD disease due to a point mutation in a mtDNA-encoded tRNA gene. The spectrum of clinical phenotype incudes seizures, myoclonus, ataxia, mental deterioration and myopathy. RRFs are demonstrated in all muscle biopsies. Another characteristic finding is the presence of numerous COX negative fibers. Matsuoka reported the amount of COX negative fibers between 5.1-37.2% [53].

NARP (Neuropathy, Ataxia, Retinitis Pigmentosa) and MILS (Maternally Inherited Leigh’s Syndrome)

Both NARP and MILS are associated with the mtDNA T8993C point mutation, the clinical phenotype depends on the mutation load. Although the central nervous system pathology is severe, the skeletal muscle pathology is unremarkable. Neurogenic muscle atrophy might occur in muscle biopsy sample, but the typical RRFs, or COX negative fibers are usually absent [54,55].

LHON (Leber Hereditary Optic Neuropathy)

Skeletal muscle involvement is unusual in LHON, as well as skeletal muscle biopsy is normal both in light microscopic and electron microscopic level [56].

MtDNA Depletion Syndromes

Clinically, mtDNA depletion syndromes are usually infantile-onset disorders with serious disease course and fatal outcome. Phenotypically hepatopathy, encephalopathy and cardiomyopathy occur. In spite of the remarkable biochemical RC defect the pathological changes are usually mild. Non-specific accumulation of mitochondria can be seen, but RRFs are absent and COX activity usually is preserved in biopsy sample, but scattered COX negative fibers might occur [57].

SUMMARY

In the era of molecular genetics muscle biopsy still plays an important role in the diagnostic procedure of MDs to confirm the diagnosis, or to guide genetic tests.

During the evaluation of muscle sample we carefully have to take into account the clinical phenotype and the patient’s age, because mitochondrial alterations are variable both in type and amount. In childhood cases RRFs are usually absent, or few, but they are common in adult patients.
We have to take into consideration that in elderly mitochondrial alterations are common in muscle biopsies.

The absence of typical changes (e.g. RRF, COX negative fibers, SSVs) can be possible in MDs, especially in childhood cases and in single organ diseases (e.g. LHON), and in opposite, secondary mitochondrial changes might develop in the advanced stage of various neuromuscular disorders.

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References


