Diagnosis of mitochondrial cytopathies using optical and electron microscopy on skeletal muscle

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Mitochondrial cytopathies are a group of diseases caused by an oxidative phosphorylation failures the final step, of energetic mitochondrial metabolism, with the subsequent ATP synthesis deficiency. These pathologies are produced by mitochondrial (mDNA) and/or nuclear DNA (nDNA) mutations. Clinical manifestations are quite diverse, being the most representative: ptosis, ophthalmoplegia, pigmentary retinopathy, optical atrophy, deafness, mild facial weakness, proximal weakness of lower extremities (muscular fatigue), exercise induced cramps, myoglobinuria, metabolic acidosis (lactic acidosis), stroke-like episodes, convulsive crisis, dementia, hypotonia, mental regression, and cardiomyopathy. Some of these manifestations are grouped into specific syndromes known as mitochondrial encephalomyopathy, lactic acidosis, stroke-like episodes (MELAS), myoclonic epilepsy with ragged-red fibers (MERRF), progressive external ophthalmoplegia (PEO), Kearns-Sayre syndrome (KSS), Pearson Syndrome (PS), Leber hereditary optic neuropathy (LHON), mitochondrial neurogastrointestinal encephalopathy (MNGIE), Neuropathy, ataxia, and pigmentary retinopathy (NARP), Sensory Ataxic Neuropathy, Dysarthria, and Ophthalmoplegia (SANDO). Muscle biopsy is a useful complementary investigation in the diagnostic workup of mitochondria disorders. These stains allow us to see the morphology and alterations of the muscle fiber and with the enzymatic reactions we can differentiate which of the complex are deficient enzymes.

Keywords: mitochondrial cytopathies, skeletal muscle, disease

1. Introduction

Mitochondrial diseases are caused by mutations in mitochondrial or nuclear genes, or both and most patients do not present with easily recognizable disorders (Sundaram et al 2004). The term mitochondrial cytopathy is used to describe a number of diseases caused by a disturbance in one or more of the mitochondrial metabolic pathways (Janet S et al, 2003). Mitochondrial disorders present the widest clinical spectrum among the metabolic diseases. These disorders are characterized by dysfunction in multiple organs or systems such as, brain, liver, kidney, muscle, heart, retina, bone marrow, pancreas and peripheral nerve, with an extensive variability of clinical presentation (Gerald P, et al 2013). Mitochondria are localized in the cytoplasm of most eukaryotic cells, are ubiquitous organelles and are the major generators of cellular ATP by oxidative phosphorylation (OXPHOS). Electrons generated by oxidation of fat and carbohydrates are transferred to oxygen via the redox components of the respiratory chain (complexes I – IV), forming water. Protons are pumped across the inner membrane from the matrix to the inter-membrane space forming an electrochemical gradient which is used by the ATP synthase (complex V) to synthesize ATP (Greaves and Taylor 2006).

The term of mitochondrial pathology applies primarily to the oxidative phosphorylation defects related, to caused by alterations in the sequence of mitochondrial DNA (mtDNA) or nuclear DNA. In fact mitochondria contain their own genome, derived from the fact that these organelle with bacterial origin have been integrated into the eukariotic cells long time ago during the evolution. Only 40% of adult cases and 10% of children have one of the mutations reported. mtDNA is a circular 16,569 base pairs molecule (Dimura et al, 2005; Tuppen et al, 2010; Chan and Nills 2011).

This genome contains information for 37 genes comprising two ribosomal RNA (rRNA), 22 transfer RNAs (tRNA) genes and genes coding for the 13 structural proteins of the four complexes (seven subunits of complex I, one of complex III, 3 2 complex IV and V complex) oxidative phosphorylation. Only the complex II is completely encoded by the DNA nuclear10. Oxidative phosphorylation enzyme complex consists of five protein-lipid located in the mitochondrial inner membrane. The mitochondrial myopathies can result from abnormalities of mtDNA or nDNA. Point mutations of mtDNA affect the protein-coding regions of the genome and tRNA genes which alter intra-mitochondrial protein synthesis. Single large-scale deletions are typically due to sporadic events and are usually not inherited, although inherited deletions have been described (1). Depletion (loss of mtDNA) and multiple deletions are typically secondary effects of alteres mtDNA maintenance, related to mutation of nDNA-encoded mitochondrial proteins (Pfeffer and Chinnery, 2013).
2. Clinical features

The prevalence of mitochondrial disorders as a whole is approximately 1 in 10,000 (5), although the carrier frequency of mtDNA mutations is about 1 in 200 [Elliott et al. 2008; Schapira and Slvatore 2002].

Chronic progressive external ophthalmoplegia (CPEO) is one of the most common manifestations of mitochondrial myopathy, presenting with slowly progressive ptosis and multidirectional limitation of ocular movements, affecting maximally vertical gaze. Gaze is often dysconjugate; however, diplopia is usually absent or transient. Kearns-Sayre syndrome (KSS) is the most severe. CPEO is variant and recognized by a triad of features, pigmentary retinopathy progressive external ophthalmoplegia and heart block. [Berenberg et al, 1977; Schapira and Slvatore 2002]. Other cardiac conduction defects described in KSS include left anterior hemiblock, right bundle branch block and type II atrioventricular block [Roberts et al, 1979]. The diagnostic criteria quote an age of onset below 20 years but improved identification of cases has revealed later onset in some cases.

In pure CPEO ophthalmoplegia and ptosis may be the only manifestations of this mitochondrial myopathy [Petty et al, 1986; Johns et al, 1993; Schapira and Slvatore 2002]. Mitochondrial encephalomyopathy, lactic acidosis and stroke like episodes (MELAS), the clinical features include psychomotor retardation, ataxia, cognitive impairment, deafness, DM, and limb weakness, optic atrophy, movement disorders, peripheral neuropathy, myoclonus, pigmentary retinopathy, spasticity [Yoneda et al, 1989; Schapira and Slvatore 2002]. Myoclonic epilepsy and ragged red fibers (MERRF) the core clinical features are of myoclonus ataxia and seizure, myoclonus is often the presenting symptom and may be induced by action, nose, or photic stimulation. Seizure types are variable but include drop attacks, focal seizure and photosensitive tonic-clonic seizures [Hammans et al, 1993; Berkovic et al, 1989; Schapira and Slvatore 2002]. Neurogenic muscle weakness, ataxia pigmentary retinopathy (NARP) has also been associated with polymorphonuclearly confirmed obstructive sleep apnea, which respond to tracheostomy, ocular manifestations have been described as typical pigmentary retinopathy, peripheral neuropathy, ataxia, seizures and dementia including migraine and mental retardation [Holt et al, 1990; Ortiz et al, 1993; Puddu et al 1993; Schapira and Slvatore 2002; Turnbull HE et al, 2010; DiMauro et al, 2011]. Mitochondrial neurogastrointestinal encephalopathy (MNGIE) include neurothy peripheral neuropathy, gastrointestinal dysmotility, gastrointestinal disease most commonly manifest as recurrent nausea, vomiting, and diarrhea. Leber hereditary optic neuropathy (LHON) this is a maternally inherited bilateral acute or subacute painless optic neuropathy, the majority of cases result in severe and permanent vision loss [Harding et al, 1994; Schapira and Slvatore 2002; Turnbull HE et al, 2010; DiMauro et al, 2011]. Pearson Syndrome (PS) is a mitochondrial disorder, affected infants manifest a refractory, transfusion-dependent sideroblastic anemia, vacuolization of hematopoietic precursors, and characterized by exocrine pancreatic insufficiency due to fibrosis acinar atrophy resulting in malabsorption and chronic diarrhea and poor growth or failure to thrive [Pearson et al, 1979; DiMauro et al, 2011]. The last, although frequently encountered, may be absent in some cases of Pearson syndrome. The condition is now known to be a rare, multisystemic, mitochondrial cytopathy with anemia, neutropenia, and thrombocytopenia, as well as variable hepatic, renal, and endocrine failure. Death usually occurs early in life (before age 4 years). The most common causes of death are lactic acidemia (which may be triggered by infection) and liver or renal failure (Blaw; 1990), the features of this progressive disorder may change over time [Pearson et al, 1979; DiMauro et al, 2011].

3. Genetics of mitochondrial disorders

Each nucleated cell has only one nDNA, but it has multiple mtDNA copies. Every mitochondrion has 5 to 10 mtDNA copies, and each cell has hundreds to thousands mitochondria. The mtDNA contains information on both acid nucleic strands and its genetic code differ from the nDNA code for some triplets. The mtDNA mutation rate is 100 to 1000 times higher than that for nDNA genes because of high metabolic activity and limited repair mechanisms. The mtDNA segregation is replicative or stochastic, and is the molecular base for heteroplasmic and for the threshold expression of phenotype. The heteroplasmic, a unique aspect of mtDNA-associated genetics, is a mix of mutant and wildtype mtDNA molecules in coexistence inside the same mitochondrion. By contrast, homoplasmic is the presence of mtDNA molecules having the same genotype. Heteroplasmic mutations often have a variable threshold, a level in which the cell can tolerate defective mtDNA molecules. (Alston et al, 2016).

With only 13 OXPHOS polypeptides encoded by mtDNA, the bulk (at least 70) of the OXPHOS sub-units are encoded by the nuclear genome. There are also several nuclear gene products that regulate mitochondrial gene expression. In figure 1 is shown the schematic of the OXPHOS complexes, their component subunits and associated ancillary factors. (Smetitink et al, 2001)

Defects in nDNA genes can be inherited in an autosomal or X-linked manner, whereas, mtDNA is maternally inherited. Mitochondrial diseases can result from mutations of nDNA genes encoding subunits of the electron transport chain complexes or their assembly factors, proteins associated with the mitochondrial import or networking, mitochondrial translation factors or proteins involved in mtDNA maintenance. MtDNA defects can be either point mutations or rearrangements. (El-Hattab et al, 2016). In figure 2 genetic defects leading to mitochondrial dysfunction are represented.
Fig. 1. Schematic of the OXPHOS complexes, their component subunits and associated ancillary factors. Multimeric protein complexes I-IV shuttle electrons along the respiratory chain, facilitated by the reduction of co-factors Coenzyme Q10 (Q) and cytochrome c (cyt c). Electron transfer is coupled to the transfer of protons (H+) across the inner mitochondrial membrane to generate a proton motive force which is used by Complex V (ATP synthase) to synthesise ATP. Characterisation of OXPHOS complexes has identified the constitutive subunits that are either mtDNA or nuclear-encoded, and many of the nuclear-encoded proteins involved in complex assembly, biogenesis or ancillary function; genes in which mutations have been identified are shown in bold and the first report of disease-causing mutations is referenced accordingly in blue.
**Fig. 2. Genetic defects leading to mitochondrial dysfunction** (MELAS: mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes; MERRF: myoclonic epilepsy with ragged-red fibers).

### 4. The procedure of muscle biopsy

Medical records were obtained through physical examination of manifestations and complimentary studies depending on the suspected diagnosis, i.e. electromyography, serum creatine kinase (SCK) levels, lactate levels, neuroimaging studies and other ancillary investigations. Open muscle biopsy was performed under local anesthesia. Samples were frosted in isopentane cooled with liquid nitrogen. Frozen muscle samples where cut on cryostat obtaining 8 μm width transverse section. The sections were stained with hematoxylin-eosin (H-E), Gomori’s modified trichrome (T-G), Schiff periodic acid (PAS) and oleic red (Gold). Enzymatic reactions such as reduced nicotinamide adenine dinucleotide, dhydrogenase-tetrazolium reductase (NADH-TR), (NADH) and cytochrome oxidase (COX) were performed for electron microscopy, a routine protocol was used.

### 5. Histological and Histochemical Stain

#### 5.1 Haematoxylin and eosin (H&E)

1. Place sections in Harris haematoxylin for 10 minutes
2. Blue in Scott’s tap water for 5 minutes
3. Place in 1% eosin for 5 minutes (or longer)
4. Wash quickly in distilled water
5. Dehydrate rapidly in ascending alcohol series
6. Clear, and mount in synthetic resin

#### 5.2 Modified Gomori Trichrome (m-TG)

1. Stain in Harris’s haematoxylin for 10 minutes
2. Rinse in distilled water
3. Stain in Gomori Trichrome mixture for 30 minutes (until green)
4. Rinse in acidified water

(if result are too red differentiation in 0.2% acetic acid can be included at this stage, followed by a rinsed in water)
5. Dehydrate rapidly in ascending alcohol series
6. Clear, and mount in synthetic resin

5.3 Periodic acid-schiff technique (PAS) for glycogen
1. Fix sections in Carnoy 2 for 5 minutes
2. Wash in distilled water
3. Incubate with periodic acid (0.5 g crystallized + 100 ml distilled water) for 10 minutes
4. Wash in distilled water
5. Place in Shiff’s reagent for 30 minutes
6. Wash in distilled water
7. Dehydrate in ascending alcohol series
8. Clear, and mount in synthetic resin

5.4 Oil red O (ORO) stain for lipid
1. Fix sections in Baker solution at 4°C for 30 minutes
2. Rinse section in tap water
3. Transfer to ORO stain for 15 minutes at 37°C closed
4. Wash in distilled water
5. Counterstain in hematein for 1 minute
6. Rinse in tap water to blue
7. Mount in aqueous mountant

5.5 DPNH NADH-Tr
1. Incubate for 30 minutes at 37 °C, in the incubation solution (Buffer TRIS 0.2 M, pH7.4, Nitrotetrazolium 1 gr/ml, DPNH (NADH-TR) 4mg).
2. Rinse in distilled water
3. Mount in aqueous mountant

5.6 Cytochorme oxidase (COX)
1. Incubate fresh frozen sections in the mixture (pH 7.4 phosphate buffer, and cytochrome C diaminobenzidine) filtered before placing the slides, for 4 hours at 37°C.

It is recommended to wear gloves when reagents are weighed and when the reaction is handled
2. Rinse in distilled water
3. Mount in aqueous mountant

Digital images were collected again in a Nikon ECLIPSE E200 microscope, using the Q-capture Pro 7 software

5.7 Electron Microscopy technique
1. muscle was fixed in 2.5% glutaraldehyde for 1 hour, three washes with phosphate buffer pH 7.2.
2. Post-fixation with osmium te-traoxido 0.5%, SO₄ for 1 hour
3. 3 washes with buffer
4. Gradual dehydration with Ketones 50 %, 60%, 70%, 80%, 90%, for 10 minutes either one and two changes of 100%
4. Subsequently a pre-inclusion of 1:1 ketone/hypoxic resin for 18 hours
5. The inclusion was polymerized at 60 ° C overnight
The semi-thin cuts of 1 micron thick were stained with toluidine blue and finally ultra-thin sections of 60-90 nm thick which were stained with uranyl acetate for 20 minutes and lead citrate for 10 minutes, the photos were taken in a JEOL 1400 plus microscope. This electron microscopy was bought by CONACyT Grant 226201.

6. Results
Optical microscopy allowed the detection of mitochondrial cytopathy cases. Through H-E we identified fascicle morphology, fiber shape and size, connective tissue, and nuclear position including internalization or centralization. TG stain to discriminate fibers type 1 from type 2, mitochondrial network and the presence of ragged-red fibers (RRF), as a result of altered mitochondrial accumulation in the subsarcolemmal regions of muscle fibers. PAS staining PAS surcharge in type 1 fibers (Fig. 3) ORO staining disclosed excess of lipid in droplets more abundantly present in type 1 fibers and especially in RRFs, NADH-TR helped distinguishing between fiber types, and disclosed abnormal mitochondrial accumulations known as “ragged blue fibers” (type 1 fiber) corresponding to alteration in the complex I of the respiratory chain. COX evidenced the presence of pale and, or negative or ghost type 1 fibers showing complex
IV deficiency (Fig. 4). By means of electron microscopy we found sub-sarcolemmal, enhanced mitochondrial accumulation in muscle or great size mitochondria with mitochondrial crestae alterations often showing mitochondrial paracrystalline inclusions (Fig. 5).

**Fig. 3** (A) H-E, B) TG, C) PAS: Muscle biopsy from a control patient shows preserved fibres. Abnormalities on skeletal muscle biopsy in patients with mitochondrial citopathy showing: (D-G) haematoxilin and eosin, (E-H) Modified Gomori Trichrome the sub-sarcolemmal accumulation of mitochondria and ragged red fibres can see (arrows), (F-I) PAS glycogen accumulates in the muscle fibres (arrows). 10X and magnification 40X.
Fig. 4 (A) Oil red O (ORO), B) NADH, C) COX: Muscle biopsy from a control patient shows preserved fibres, Abnormalities on skeletal muscle biopsy in patients with mitochondrial citopathy showing: (D-G) Oil red O intracellular lipid can see type 1 fibres (arrows), (E-H) NADH type I “fibers blue”, seceraal with sarcolemmal oxidative enzyme-positive staining (dark), representing accumulation of mitochondria (arrows), (F-I) Cytochrome c oxidase histochemistry (COX) deficient histochemistry showing a mosaic pale (arrowhead), negative or ghosts fibers (*) can see in patients with mtDNA disorders (arrows), 10X and magnification 40X
Fig. 5. Cross section of muscle; electron microscopy microphotography showing mitochondrial crystalline inclusions and glycogen (G) A), the mitochondria present altered crests, accumulation of the glycogen can see B) Lost of mitochondrial crests C). Alteration of the mitochondria’s and mitochondrial accumulation D). Bar 2.0 µm and 500.0 nm.

7. Conclusions

Mitochondrial cytopathies are multisystemic disorders, caused by mDNA and/or nDNA mutations, with a great variability in symptoms and multiple alterations in paraclinical through studies. The study of skeletal muscle biopsy comprising both optical and electron microscopy support the diagnosis and enable a clear orientation of biochemical and mitochondrial genetic studies.

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