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Diagnosis of Myopathies Using Histology, Histochemistry, Immunohistochemistry and Electron Microscopy: A Single Center Experience

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Abstract

Background: Skeletal muscle biopsy is important for the diagnosis of motor unit disorders, systemic diseases and metabolic disorders. In some cases, routine histopathologic methods are not conclusive and histochemistry, immunohistochemistry and even an electron microscopic study are required. In this study, we describe our experience in the diagnosis of myopathies, considering all of the above-mentioned methods.

Methods: During a period of 18 months, 43 specimens of patients with the impression of myopathy were submitted to the Pathology Department and were evaluated with H & E and histochemical stainings (PAS, Oil red O, ATPase, NADH-TR, Gomori Trichrome), immunohistochemistry (IHC) for dystrophin and electron microscopy. Three specimens were excluded from the study because there were only adipose tissues and no adequate muscle was present for evaluation.

Results: Twenty three (57.5%) males and 17 (42.5%) females with a mean age of 34 years were evaluated. The results were as follows: Becker's muscular dystrophy (5 cases, 12.5%), Duchenne's muscular dystrophy (3 cases,7.5%), fascioscapulohumeral dystrophy (3 cases, 7.5%), limb girdle dystrophy (2 cases, 5%), polymyositis (6 cases, 15%), dermatomyositis (2 cases 5%), McArdle's disease (1 case, 2.5%), hypothyroidism myopathy (1 case, 2.5%), type 2 atrophy secondary to drugs and systemic diseases (2 cases 12.5%), congenital myopathy (2 cases 5%), McArdle (1 case 2.5%), unclassified myopathy (2 cases, 5%), and normal muscle biopsy (8 cases, 20%). Although a genetic study was not available to confirm the diagnosis of cases such as fascioscapulohumeral myopathy, the diagnosis was made after putting all of the findings together including clinical presentation, family history, NCV, EMG, etc.

Conclusion: In the cases with no definite diagnosis by the histology, histochemistry and IHC, we should perform an EM study to find out the distinct ultra-structural changes which can be diagnostic for some muscle disorders. EM study in conjunction with light microscopy of muscle biopsy could be very helpful in establishing the diagnosis of some types of myopathies.

Keywords: Muscle biopsy; Muscle disorder; Histochemistry; Immunohistochemistry; Electron microscopy

Introduction

Skeletal muscle biopsy is important for the diagnosis of the motor unit disorders such as inflammatory myopathies and dystrophies, systemic diseases such as vasculitis and metabolic diorders such as glycogenosis.[1] Some disorders can be diagnosed by routine section stained by haematoxylin and eosin (H&E) under a light microscope which clearly shows the overall structure of the tissue in relation to the fibers, nuclei, fibrous and adipose tissue, the presence of inflammatory cells, and vascular and neural components.[2] However, histochemistry is necessary in some conditions including modified Gomori trichrome,

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Congo Red, nicotinamide adenine dinucleotide reduced (NADH-TR), succinic dehydrogenase (SDH), cytochrome oxidase, acid phosphatase; periodic acid-Schiff (PAS), oil red O, adenosine triphosphatase (ATPase) preincubated at PH 9.4, 4.6 and 4.3, immunohistochemistry such as dystrophins, sarcoglycans and merosin, and even an electron microscopic study.[1,3,4] The only routine light microscopic method (H&E stain) is not conclusive in the diagnosis of many skeletal muscle disorders.[2-4] In this study, we describe our experience in the diagnosis of myopathies, considering H&E staining, histochemistry, IHC, and electron microscopy (EM).

Materials and Methods

The skeletal muscle biopsies of the patients referred to Motahari Clinic of Shiraz University of Medical Sciences, and Clinic of Neurology and Rheumatology, were the material of this study. Forty three patients with the impression of myopathy were selected in a period of 18 months; the selection was based on history, physical examination, laboratory investigation, and Electromyography (EMG)/ nerve conductive velocity (NCV). All of the muscle biopsies were taken from the quadriceps muscle with approximately 2×1×0.5 cm in size.

The specimens were immediately submitted to the pathology laboratory and all of them were received fresh. They were then divided into three portions as follows:

i. Transverse and longitudinal sections were fixed in 10% buffered formalin for H&E staining and IHC, ii. Transverse and longitudinal sections were held in 3% cacodylate buffered glutaraldehyde (pH of 6.9 at 4ºC) for electron microscopic study, iii. A transverse section was placed on cork disc with cross orientation by a small amount of OCT mounting medium (Merck) around the base of the specimen. The cork with its specimen was then inverted into the liquid phase of isopentane, previously cooled in liquid nitrogen, and then this bottle was rapidly immersed in liquid nitrogen about 20 seconds (without mixing with isopentane) until the specimen was cooled to -160ºC , being completely frozen. Before sectioning, the cork was also frozen into a microtome chuck with OCT. For each specimen, about 15-20 slides of frozen sectioning with 8 µm thickness in a cryotome (Leica) at -23 to -25ºC were prepared for histochemistry staining including PAS, Oil red O, ATPase, NADH, and Gomori Trichrome.

Haematoxylin and eosin (H&E), PAS, Gomori trichrome, oil red O, NADH, and ATPase were performed by routine conventional methods.[5] IHC for dystrophin was done on paraffin embedded tissues.[6] Positive controls were normal skeletal muscle and the staining pattern was membranous.[6] For electron microscopy, a small fragment of the muscle specimen with cross and longitudinal sections were used for each patient and a normal muscle specimen was included as the negative control. Immediately after the excision, the specimen was fixed in 3% cacodylate buffered glutaraldehyde post-fixed in osmium tetroxide, dehydrated through epoxy resin (agar 100) at 65ºC for 24 hours. The semithin 1 µm –thick sections were stained with toluidine blue and examined under a light microscope. The ultrathin 70 nm thick sections were put on copper grids and stained with uranyl acetate and lead citrate and examined under an electron microscope LEO 906.[7]

Results

During 18 months, 43 specimens of patients with the clinical impression of myopathy were submitted to the pathology lab. Three specimens were excluded from the study because there were only adipose tissues or minute fragments of muscle fiber (inadequate for diagnosis). Altogether, 23 (57.5%) males and 17 (42.5%) females aged 6 to 70 years (mean = 34 years) were included in the study and were evaluated with histological, histochemical, immunohistochemical and an electron microscopic study. The results are shown in Table 1 showing that we had 13 cases of muscular dystrophy, all of whom were diagnosed by putting all the clinical and paraclinical findings together.

In this study, we diagnosed Becker's muscular dystrophy (BMD) in 5, Duchenne's muscular dystrophy (DMD) in 3, fascioscapulohumeral in 3 and limb girdle muscular dystrophy in 2 patients. Histopathologic study of H&E sections was in favor of dystrophy including severe random variation of the muscle fiber size, internal nuclei, clusters of necrotic fibers, fiber splitting, and fibrosis. IHC staining for dystrophin was absent in DMD, decreased in BMD and normal in other dystrophies. Histochemical findings were non-specific and showed type I atrophy in some of the cases.

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Table 1: Incidence of myopathic disorders with sex and age in 40 cases

Myopathic disorders	No	%	Male	Female	Mean age±SD (years)
Becker muscular dystrophy	5	12.5	4	1	36.5±10.0
Duchenne muscular dystrophy	3	7.5	2	1	19.0±10.0
Facioscapulohumeral dystrophy	3	7.5	3	-	38.5±16.5
Limb girdle dystrophy	2	5.0	-	2	36.0±12.5
Polymyositis	6	15.0	3	3	36.0±15.0
Dermatomyositis	2	5.0	1	1	39.0±21.0
Type 2 muscle atrophy	5	12.5	2	3	37.0±21.5
Congenital myopathy	2	5.0	2	-	10.5±6.0
McArdle's disease	1	2.5	1	-	30.0
Hypothyroidism myopathy	1	2.5	1	-	52.0
Unclassified myopathy	2	5	2	-	43.5±36.5
NSPC	8	20	2	6	33.5±15.0

EM findings in the patients who were clinically diagnosed as fascioscapulohumeral muscular dystrophy showed segmentation of the nuclei with irregularity of the nuclear membrane; polar aggregation of mitochondria, glycogen and a few fat droplets around the nucleus and occasionally between the myofibrils, and splitting and narrowing of the myofibrils (Figure 1).

We also diagnosed 2 patients as limb girdle muscular dystrophy (LGMD), EM findings of which showed filamentous body at the periphery of the fiber surrounded by mitochondria and collagen fiber over the basement membrane (Figure 2).

EM findings of patients with BMD and DMD showed indentation of nuclear membrane and

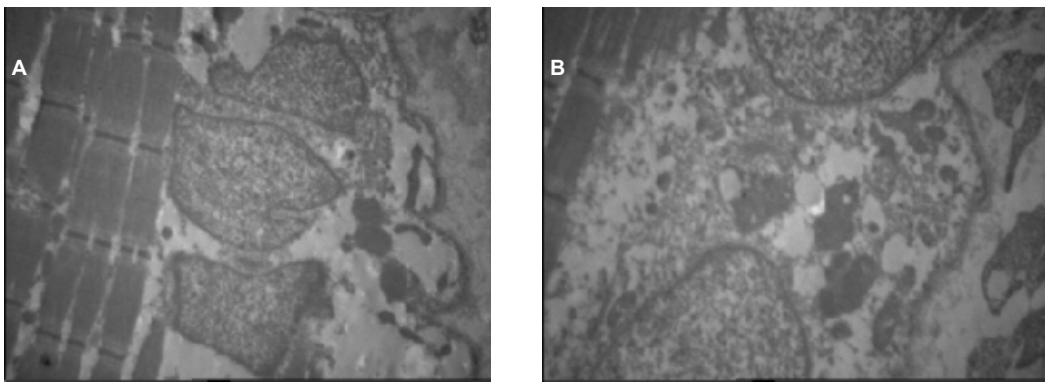

Fig. 1: Electron micrograph of a case of FSHD showing: (A) Segmentation of nuclei with irregularity of the nuclear membrane, Polar aggregation of mitochondria, glycogen and (B) A few fat droplets around the nucleus and occasionally between the myofibrils (uranium acetate, lead citrate).

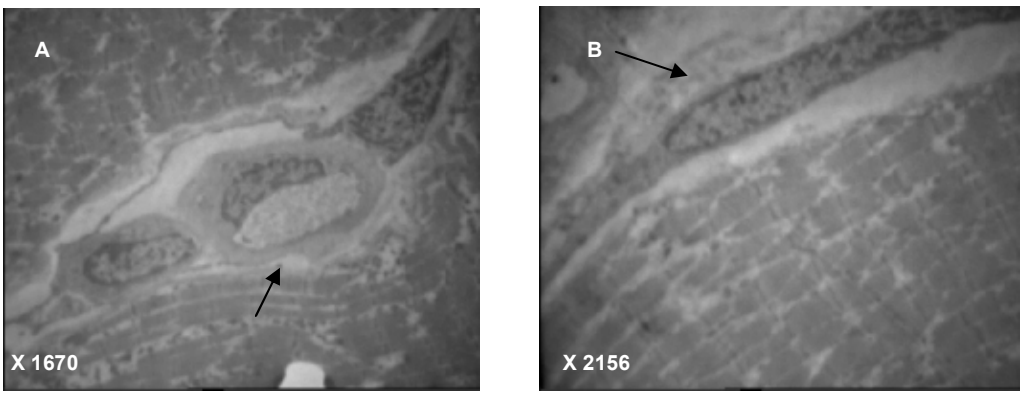

Fig. 2: Electron micrograph of a case of LGMD showing: (A) Filamentous body at the periphery of the fiber. (B) Collagen fiber over the basement membrane (uranium acetate, lead citrate)

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subnuclear chromatin condensation, subsarcolemmal accumulation of mitochondria in an area with loss of myofibrils and adjacent to a nucleus or between myofibrils with variability in size (Figure 3) and replicated capillary basement membrane (Figure 4). Unfortunately, no genetic study was available for confirmation.

In the population under the study, there were 8 patients who were clinically diagnosed as inflammatory myopathy and the diagnosis was confirmed by serologic studies. Histologic findings were almost diagnostic including diffuse endomysial, perifasicular and perivascular infiltration of lymphocytes. IHC for dystrophin was normal in all the cases. EM showed spherical nuclear inclusions surrounded by haloes in the endothelial cells (Figure 5). Histochemical staining was completely nonspecific.

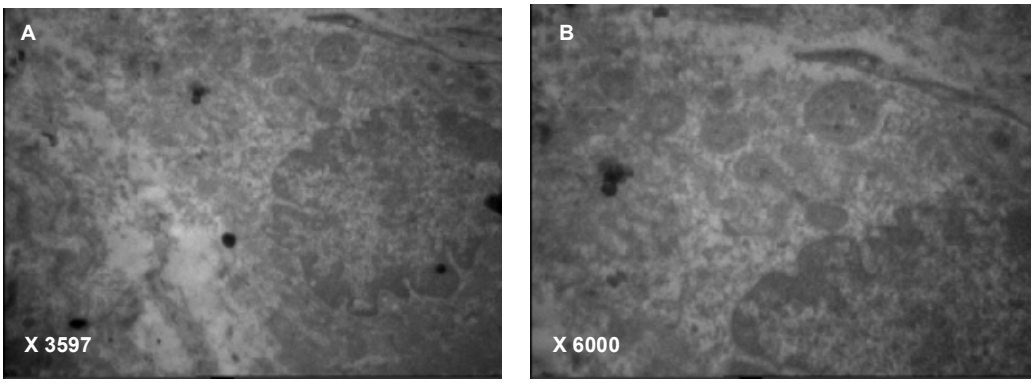

Fig. 3: Electron micrograph of a case of BMD showing (A) indentation of nuclei, subnuclear chromatin condensation & (B) subsarcolemmal accumulation of mitochondria (uranium acetate, lead citrate)

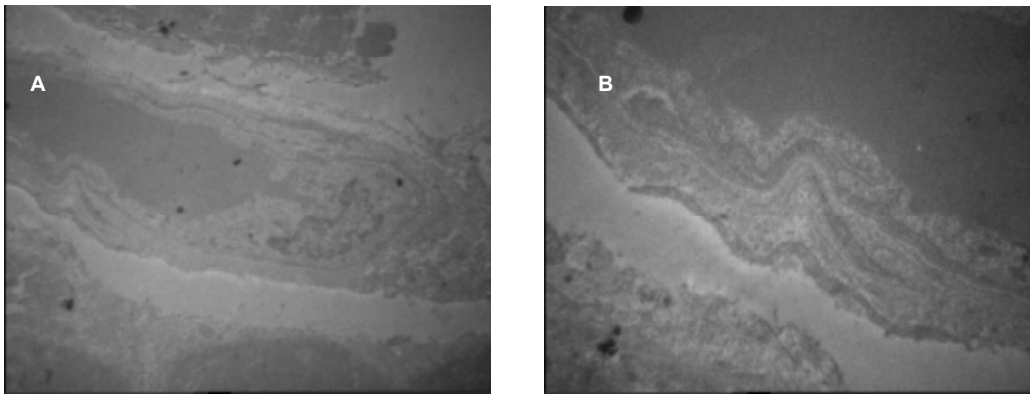

Fig. 4: Electron micrograph of a case of BMD showing (A&B) reduplication of capillary basement membrane (uranium acetate, lead citrate)

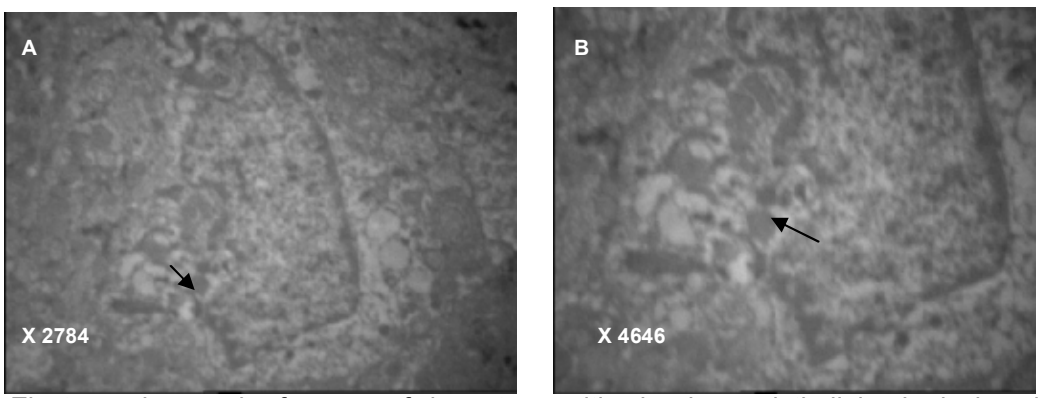

Fig. 5: (A&B) Electron micrograph of a case of dermatomyositis showing endothelial spherical nuclear inclusions surrounded by haloes (uranium acetate, lead citrate)

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In a patient with clinical impression of Mc Ardle's disease, H&E, histochemistry and IHC were completely normal but EM showed granules of glycogens in the cytoplasm of the myofibers with vacuolization and some of vacuoles contained few granules of glycogen (Figure 6). Five patients with pure type II atrophy had the history of corticosteroid consumption for diseases such as rheumatoid arthritis.

In 2 patients who were labeled as congenital myopathy, a significant number of fibers with internal nuclei, severe variation in size and shape of the fibers, and endomysial fibrosis were found. IHC was normal for dystrophin; the tissue was not enough for interpretation of EM study in one case and in the other just showed nonspecific findings of myopathy. One of the cases with hypothyroidism showed type II atrophy with normal EM and IHC. Moreover, there were 2 patients for whom no specific diagnosis was made despite all of the above-mentioned methods, so they were labeled as unclassified myopathy. EM, IHC, histochemistry and H&E stains of 8 patients were completely normal.

Discussion

Muscular dystrophies were the most common type of myopathies (32.5%) and BMD was the most common type of dystrophy in our study, while the incidence of DMD was approximately 10 times more than that of DMD in other studies.[3,4] This may be due to the death of DMDs before a definite diagnosis by a clinician or due to the limited number of cases in this study.

Although DMD and BMD are recessive X-linked disorders, females with a chromosomal translocation or female carriers, according to the Lyon hypothesis, are affected as an affected male.[2] We differentiated DMD from BMD with IHC for dystrophin (absence of sarcolemmal immunostaining in DMD and incomplete sarcolemmal immunostaining in BMD) with no ultra-structural difference.

Three cases were suspected with facioscapulohumoral dystrophy (FSHD) among our dystrophy cases based on histopathology (presence of very small fibers scattered among the larger fibers in H&E stain), IHC for dystrophin, clinical information, and electron microscopy (EM). We suggested the diagnosis of FSHD in another case by EM.

The ultra-structural findings of FSHD were splitting and narrowing of myofibrils, segmentation of the nuclei and polar aggregation of mitochondria, glycogen and a few fat droplets around the nucleus and areas between the myofibrils.[2]

Both cases of limb girdle dystrophy were suspected with the presence of filamentous body at the periphery of the fibers and collagen fiber over the basement membrane in electron microscopy.[2] A good history of muscular involvement areas and clinicopathologic correlation are very effectual in the diagnosis of different kinds of dystrophies. Despite the high incidence of inflammatory myopathies in previous studies, it was the second most common disease. Polymyostis was the most common inflammatory myopathy.

According to the previous reports, polymyositis is the most common inflammatory myopathy in adults[3,8] like what we found in our study. In this study, EM was not helpful for the diagnosis of inflammatory myopathies. Of course, the presence of endothelial spherical nuclear inclusions surrounded by haloes in EM confirmed the diagnosis of dermatomyositis.[2]

Histochemistry and electron microscopic studies are important to rule out inclusion body myositis.[9] A

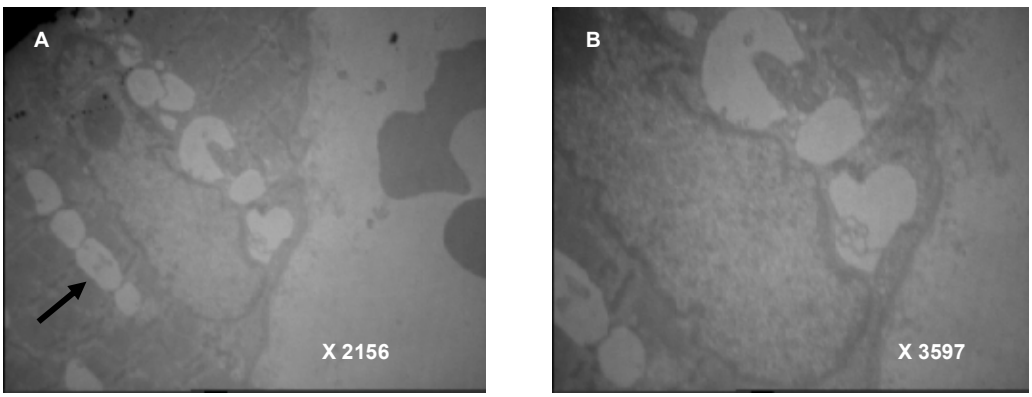

Fig. 6: (A&B) Electron micrograph of a case of Mc Ardle's disease showing granules of glycogens in the cytoplasm of the myofibres with vacuolization (uranium acetate, lead citrate)

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good history such as the age of 50 years and unresponsiveness to therapy for PM is useful for the diagnosis.[1] We did not have any case of inclusion body myositis in our patients. The most common cause of type 2 muscle atrophy in our study was steroid therapy, whereas in the previous reports alcohol was claimed to be the most common cause of type II atrophy.[2] This may be related to low alcohol intake in Iran. The presence of type 2 fiber atrophy in two cases of rheumatoid arthritis could be due to autoimmune process, or steroid therapy. A case of ovarian cancer also showed type 2 atrophy, considered as a paraneoplastic disorder. H&E staining in these cases showed atrophic fibers and histochemical staining such as ATPase confirmed typed 2 fiber atrophy.[2] EM in these patients was not effective.

We diagnosed two cases of congenital myopathies. Both of our cases, as in the literature, were children.[2] Hypotrophy of type 1 fibers and proper history of the patients were the only clues available for suspected diagnosis.[2] However, definite diagnosis needs genetic study. EM in these patients was not useful, because in one case tissue was not adequate for definite interpretation and in the other one it showed just non-specific changes. Also, one case of McArdle's disease (glycogen storage disease type IV) was diagnosed. Histopathologic studies were non-specific in this 30 years-old male with the history of cramps during exercise. PAS and IHC for dystrophin were also normal. Accumulation of glycogen granules between myofibrils and presence of vacuolization with different-sized fibers, some of which containing glycogen in EM study, were diagnostic in this case.

A history of cramps during exercise, presence of approximately normal histopathology and accumulation of glycogen in EM or occasionally in PAS staining are consistent with diagnosis of Mc Ardle's disease.[1,2] In these cases, also myophosphorylase could be positive or normal.[1] Khaleeli *et al.* (1983) studied needle biopsies before and several months after treatment in 11 adult hypothyroid patients aged 51 to 71 years with associated muscle weakness. Abnormalities were found in 8 of 11 biopsies, the commonest finding being type 2 fiber atrophy.[10] We also distinguished a 52 year-old male patient with the history of hypothyroidism. Histochemical staining showed type 2 fiber atrophy. EM in this patient was not useful.

We categorized two patients as unclassified myopathy; histological, histochemical and ultra-structural findings in these two patients confirmed myopathic disorder including mild to moderate variation in size and shape of myofibres, vesicular nuclei, basophilic fibres, endo- and perimysial fibrosis, and necrotic fibres. However, definite classification was not possible. In previous reports, in 5000 neuromuscular patients (University of New York) 5% of the patients were categorized as non- classified myopathy despite all of the diagnostic techniques.[4] Dubowitz *et al.* made also the diagnosis of "unclassified myopathy".[2]

Muscle biopsy of twenty percent of our patients showed no specific pathologic change, the causes of which can be unsuitable selection of patients by clinicians for myopathy versus myalgia, borderline EMG findings and improper site of muscle biopsy.

According to this study, skeletal muscle biopsy is important for the diagnosis of myopathic disorders and useful diagnostic techniques are presented for each myopathic disorder. Major ultra-structural features in different muscular disorders in this investigation are as follows: i) Splitting and narrowing of myofibrils, segmentation of nuclei, and polar aggregation of mitochondria around the nucleus in FSHD, ii) Filamentous body at the periphery of fibers in LGMD, iii) Endothelial intra-nuclear spherical dense bodies, surrounded by a clear halo in dermatomyositis, iv) Indentation of nuclear membrane, subsarcolemmal accumulation of mitochondria, duplicated capillary basement membrane, and presence of mast cell in BMD and DMD and v) Granules and cytoplasmic vacuoles of glycogen in McArdle's disease.

Although these criteria are not diagnostic but can be helpful for diagnosis, especially in those cases in which other ancillary techniques are not conclusive. Based on our experience in these 40 cases and the result of the study, the following points are suggested: i) For evaluation of patients with muscular disorders, there should be a medical team consisting of a neurologist or rheumatologist and a pathologist, ii) Clinicians should collect a complete history of patients, perform physical examination and EMG/NCV for patients, and request proper laboratory tests (such as CPK, LDH) and finally, clinicians must raise appropriate impression and differential diagnosis, iii) A clinician who can be the neurologist, rheumatologist or a general surgeon must do muscle biopsy from a proper site which is moderately involved, iv) The specimens should be rapidly frozen for procedures of histochemistry to minimize fixation artifacts, v) A small sample of fresh muscle biopsy should be put in 3% glutaraldehyde for possible EM study and vi) By considering the results and economic factors, first of all we should do H&E staining, then use histochemistry

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staining and later do IHC study for DMD, BMD and other dystrophies with dystrophin.

Finally, in proper cases in which light microscopic findings are not diagnostic, we perform EM study to see distinct ultra-structural changes which is conclusive and diagnostic for some muscle disorders. In conclusion, EM study in conjunction with histochemical and immunohistochemical staining of muscle biopsy could be very helpful in establishing definite diagnosis of some myopathies.

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Conflict of interest: None declared.

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