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Review Article/บทฟื้นฟูวิชาการ

Muscle Biopsy: A Useful Tool and Approach for Diagnostic Evaluation of Muscle Disease

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Abtract

Muscle biopsy had been used to diagnose muscle disease for a long time because only a few of the disorders show adequate specific clinical features for definite diagnosis. Since the screening of numerous genes is limited to perform, muscle biopsy could be a time and cost effective tool for solving the diagnostic problems. The aim of this review article is to emphasise the importance of muscle biopsy as a useful tool and approach for diagnostic evaluation of muscle disease. The author describes the procedure for muscle biopsy, specimen handling and the microscopic findings obtained from non-enzyme (tinctorial) and enzymatic histochemical stains correlating with clinical information to achieve the best interpretation results.

Keywords: Muscle biopsy, Pathology

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Introduction

In 1865 Griesinger W. was the first who make a surgical muscle biopsy and confirmed the presence of abundant adipose tissue in a patient's large calf muscle.[1] A year later Duchenne BG., the famous neuromuscular clinician performed the muscle biopsy on a myopathic patient by using an invention called "l'emport-piece" or "Duchenne's trocar" later developed by Bergström into "Bergström's trocar".[2] Revolution in muscle pathology started in 1970 when Victor Dubowitz et al. introduced the established enzyme histochemical staining to study muscle biopsy undertaken by snap frozen technique.[3] Developments had occurred since 1980 with the utility of immunohistochemistry and specific antibodies. Subtypes of muscular dystrophies were diagnosed based on the immunohistochemical study.[3] An advance in the molecular genetic analysis since twenty-first century had brought in a magnificent progression.

Muscle biopsy is a relatively simple but strict unique technique challenges to the technologist and unique diagnosis challenges to the pathologist. A muscle sample does not proceed under conventional formalin fixed paraffin embedded methods. The fresh specimen should be carefully handled and subsequently processed in a low-temperature freezing method, which named "snap frozen technique". Then a panel of non-enzyme (tinctorial) and enzymatic histochemical stains for every muscle biopsy for complete diagnostic interpretation should be done. EM for ultra-structural study and immunohistochemical study may add valuable information in some cases.

Muscle Biopsy: Guidelines

1. Patient Selection

Complete clinical assessment of the patient is essential. Diagnosis should always base on a detailed clinical and family history, physical examination, in conjunction with special investigations such as serum enzymes, muscle imaging and electromyography. Muscle biopsy looks upon as an additional/definite confirmatory test of an underlining muscle disease. The pertinent history, physical examination and investigations of the patients can categorise as table 1.

Interpretation of a muscle biopsy depends on clinical information and pathological findings. Clinical presentation, family history and the results of any other investigations are essential to be considered. The level of serum creatine kinase (CK), is a useful indicator; Duchenne muscular dystrophy (DMD) always has a high CK, whereas levels are usually normal in congenital myopathies and neurogenic disorders. Knowledge of the muscle affected, cardiac involvement, respiratory difficulties, abnormalities in brain magnetic resonance imaging (MRI), joint contracture are also valuable. The differential diagnosis would be more accurate using a systematic analysis for interpretation. Regular multidisciplinary meeting of pathologist and clinician would give recognition key points between the biopsy and clinical features. Correlating the pathology to the clinical picture is preponderant for giving the diagnosis. For example, a striking dystrophic picture in a 7-month-old infant with a symptom of hypotonia at birth, may suggest the diagnosis of congenital muscular dystrophy. Nevertheless, a similar picture in a 15-year-old boy who was ambulant until the age of 10 would be a characteristic of Duchenne muscular dystrophy. In the same, biopsy from severely affected infant with Werdnig-Hoffmann disease or spinal muscular atrophy, SMA I, can show the same feature as biopsy in a milder case, SMA II. Evidence of pathological findings can present in some condition in the absence of any apparent neuromuscular signs, such as collagen diseases. In contrast, the muscle biopsy may show no

Table 1 Pertinent clinical information

Pertinent clinical presentation:
Clinical onset: congenital, a long standing or newly start condition Condition progression: progressive, relatively static Associated systemic conditions: heart problem, skin lesion, autoimmune disease, bulbar symptom, underlying disease Growth and development: disturbance in growth and development, walking, standing, exercise
Other helpful clinical information:
Sex and age History of exercise intolerance, myoglobinuria, second wind phenomenon Family history of neuromuscular disease History of consanguineous marriages History of medication at the time of biopsy Site of biopsy
Pertinent physical examination:
Presence of congenital facies, contracture, fracture Muscle atrophy, pseudohypertrophy, wasting, rippling, tone and reflex Affected and degree of muscle weakness
Laboratory investigation:
Serum CK level Results of EMG and nerve conduction studies MRI of affected muscle

abnormalities in myasthenia gravis or congenital myotonia. Their clinical diagnoses are confirmed by the electrodiagnostic study.

2. Muscle Selection

Muscle disease is generalised, but not all muscle is affected. Several myopathic conditions may involve certain muscle groups. Site of muscle selection is decided by clinical examination, the progression of the disease and sometimes by imaging studies. It is crucial to sample a moderately affected muscle because specimen sampling from severely affected muscle will only show fat and fibrosis. The minimally involved tissue may lack histological findings. Biopsy should be taken from muscle belly far from tendon insertion site because it can show internal nuclei, a variation of fibre sizes, whorled fibres and endomysial fibrosis mimicking myopathy.[3] Muscle which traumatised by EMG needle, sites of recent injections and previous surgery should also avoid.[4] Inaccurate interpretation may happen because certain changes such as cellular reaction in polymyositis, may have a patchy distribution. Missed diagnosis of advanced myopathic and the neurogenic process may occur due to a similar end-stage finding in both conditions. Limb girdle muscular dystrophy types (LGMDs) with cellular reaction such as LGMD2B (dysferlinopathy), LGMD2A (calpainopathy) may mimic inflammatory myopathies. Standard usual muscle biopsy sites include quadriceps, deltoid, biceps, or tibialis anterior muscles. In most of the proximal myopathies, vastus lateralis is suitable for biopsy as it is away from major vessels, and nerves.[5] Biceps and gastrocnemius are other appropriate sites for biopsy. Tibialis anterior is a biopsy site when indicated by imaging studies. Deltoid muscle biopsy is avoidable as it is a site for injections and may show no finding in all diseases. Fibre type proportion is always better to evaluate correlated with a particular biopsy site because it varies from each site. Muscle fibres measurement

should rely on own laboratory standardised normative data due to the variation in muscle fibres size among race, gender and different ages.[3] At muscle laboratory unit of Ramathibodi Hospital, we use our normative data; muscle fibre type proportion and mean size of the biceps brachii and vastus lateralis muscles.[6] A biopsy obtained from unusual sites, for example, paraspinal or neck muscles, produces problems for orientation, presence of bizarre fibres, COX-negative fibres, ragged red fibres and typing proportion evaluation.

3. Muscle Biopsy Technique

Biopsy technique can be a needle biopsy or open biopsy. Edwards and colleagues (Edwards 1971, Edwards et. al 1973, 1983) used the Bergström needle for muscle biopsy in adult patients.[7-9] Since 2008, Sewry and Dubowitz used it for infants and children under general anaesthesia.[3] Despite needle biopsies have mostly replaced open biopsies, some laboratories still use open biopsy due to the workability of the procedure and a bigger piece of muscle. Needle biopsy gains the advantage due to a rapid procedure that can be performed simply in an outpatient clinic setting. Nevertheless, it can be easily to repeat for follow-up studies. Open biopsy yields advantage due to a larger specimen adequate for additional biochemical study, immunohistochemistry or molecular analysis. A larger specimen also increases the chance of focal changes such as vasculitis or inflammatory changes. The critical point is a well collected and processed muscle minimises artefact. Clinicians should inform the muscle pathology laboratory before sending the specimen and follow the specific handling instructions. The super strict is that the muscle should be kept moist with a lightly moistened gauze with isotonic saline avoiding floating in saline and transported immediately to the laboratory for processing.[3]

4. Muscle Sample Processing

4.1. Processing protocol

A most important step is to orientate the fibres in a transverse plane first because cross sections provide most of the findings. The best way is lining all pieces of fibres in a longitudinal plane and then turn the sample on its end. The step should perform under a dissecting microscope. After that one or two pieces of fresh muscle are snap frozen in isopentane that cooled in liquid nitrogen at minus 160 °C to 180 °C and then sectioning in a cryostat at minus 20 °C.[3] Cross sections from each block can use for histological study. At our hospital, the pathologist chooses the best accurate block to proceed from stat haematoxylin and eosin stains on immediate sections. This step yields advantage result for appropriate pathological findings. The tissue process categories are in three protocols as Table 2.

Series of routine histochemical stains: haematoxylin and eosin (H&E), modified Gömöri trichrome

Table 2 Processing protocol

Protocol	Histochemistry	IHC Study Panel
I	Series of routine histochemical stains	Not required
II	Series of routine histochemical stains	Muscular dystrophy
III	Series of routine histochemical stains	Inflammatory myopathy

(mGt), nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR), cytochrome c oxidase (COX), succinate dehydrogenase (SDH), combined cytochrome c oxidase and succinic dehydrogenase (COX/SDH), periodic acid-Schiff (PAS), PAS with diastase (PASD), oil red O, myophosphorylase, alkaline phosphatase, myoadenylate deaminase (MAD) and ATPase at pH 4.3, 4.6 and 9.4.

Muscular dystrophy immunohistochemistry panel: spectrin, dystrophin(rod domain, A terminus and C terminal), sarcoglycan complex(alpha, beta and gamma), dystroglycan complex(alpha, beta and gamma), merosin, dysferlin, laminin, utrophin, MHC class I.

Inflammatory myopathy immunohistochemistry panel: CD4, CD8, CD3, CD20, CD68, MAC, MHC class I.

4.2. Biochemical and molecular analysis

For biochemical study and molecular analysis, a small fresh tissue is divided and preserved in minus 80 °C.[3,10]

4.3. Ultrastructural study

A small fresh tissue, approximately mea-suring 0.2x0.2x0.2 cm, is enough for ultrastructural study under an electron microscope (EM). A remaining tissue fixed in buffered formalin is for conventional processing.

5. Normal Muscle Pathological Finding

Normal muscle arranges in fascicles bounded by epimysium. Collagen called perimysium wrap each fascicle which composed of muscle fibres. There are arterioles, nerve bundles, venules and muscle spindles locating in the perimysium. The muscle fibres are round in a child and are polygonal in an adult. Each fibre opposes together with a very minimal intervening connective tissue called endomysium. The endomysium contains capillary blood vessels. Muscle fibres are syncytial showing peripheral nuclei with a few of internal nuclei, 3-5%. Satellite cells locate close to the periphery of fibres. Fibre type distribution is vary depend upon the muscle site. In quadriceps femoris, type proportion shows mosaic pattern due to 30:30:30% of three fibre types; I, IIA, IIB. (Figure 1A, B)

Routine tinctorial and histochemical methods

Summary of stains and their interpretation is given in Table 3.

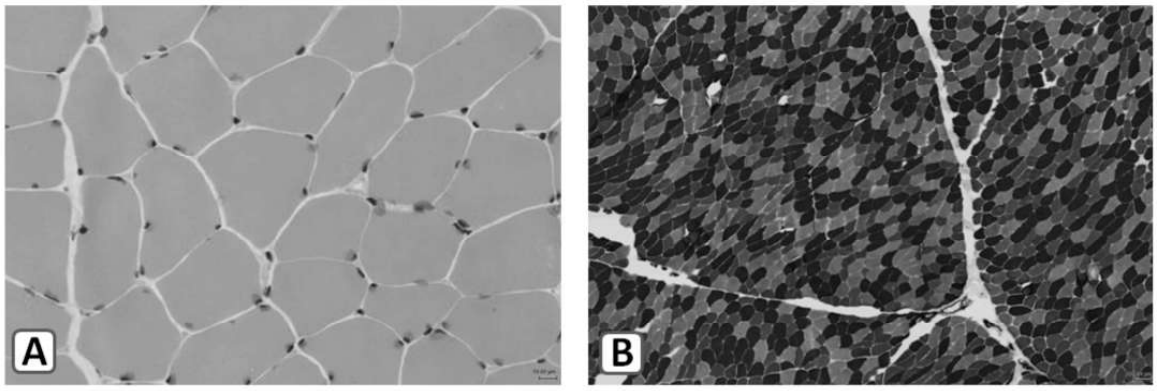

Figure 1 *Normal muscle (A) Normal muscle H&EX400 (B) Normal type proportion, mosaic pattern of three fibre types in quadriceps femoris ATPase pH 4.6X100*

Table 3 Tinctorial and Histochemical Stains

Stain	Demonstrates
Haematoxylin and Eosin	General morphology, fascicular architecture, fibre basophilia, cellular reaction, vacuolated fibres
Modified Gömöri trichrome	Ragged red fibres, rod bodies, tubular aggregate, cytoplasmic bodies, rimmed vacuoles, nuclei, myelinated fibres and connective tissue
Periodic acid Schiff(PAS) with diastase digestion(PASD)	Glycogen, PAS-positive/diastase resistant inclusions
Oil red O	Neutral lipid, intrafibre lipid droplets, interstitial adipose tissue
Acid phosphatase	Lysosomal enzymes, necrotic fibres
Crystal violet	Amyloid
Adenosine triphosphatase (ATPase pH 9.4, 4.6, 4.3)	Fibres types: I, IIA, IIB, IIC
Nicotinamide dinucleotide tetrazolium reductase (NADH-TR)	Intramyofibrillar network, cores/multi-minicores, target/targetoid fibres, lobulated fibres
Succinic dehydrogenase(SDH)	Oxidative enzyme activity
Cytochrome C Oxidase(COX)	Mitochondrial enzyme activity
COX/SDH	COX negative fibres
Myophosphorylase	Deficiency = type V glycogenosis (McArdle's disease)
Phosphofructokinase(PFK)	Deficiency = type VII glycogenosis (Tarui's disease)
Myoadenylate deaminase (MAD)	Tubular aggregate

Modified from Dubowitz V, Sewry CA, Oldfors A, Lane R. In: Muscle biopsy: a practical approach. Victor Dubowitz, Caroline A. Sewry, Anders Oldfors, Russell Lane. 4th edition. Oxford: Saunders Elsevier, 2013.

6. Histological and Histochemical Change

H&E is the most useful stain showing the general architecture of the muscle and the morphology of each fibre. Series of routine tinctorial and histochemical stains add up to the summation of pathological findings. By evaluating the different changes of muscle biopsy, and assessing these findings correlated with patient's clinical features, pathologist and clinician can obtain an accurate diagnosis.

H&E and other stains are used to find the following changes in muscle:

1. Fascicular architecture
2. Variation in fibre size and shape
3. Necrotic, regenerating and degenerated fibres
4. Atrophic fibres, small and large group atrophy
5. Nuclear internalization
6. Cellular reaction, type and distribution of inflammatory cells
7. Endomysial and perimysial connective tissue
8. Changes in fibre architecture and structural abnormalities
9. Enzyme histochemistry and deficiency of enzyme
10. Accumulation of glycogen or lipid
11. Accumulation of amyloid
12. Typical artefacts in muscle biopsy

1. Fascicular architecture

With light microscope at low magnification muscle fascicles, is first examined and assessed for the architecture, adipose tissue replacement or fibrosis. The pathological changes if any is noted. The

diffuse pattern of involvement is seen in muscular dystrophy, focal in neurogenic and patchy in inflammatory myopathies. The extent of adipose tissue infiltration and fibrosis depend upon the duration period of disease and degree of severity. Muscle fibres atrophy result to loss of normal fascicular architecture. In normal muscle, there is minimal variation in fibre size which depends on age, gender and muscle. The fibres type variation may be atrophy or hypertrophy and it may selectively involve type 1 or type 2 fibres. The involvement may be diffuse or focal.

2. Variation in fibre size and shape

The muscle fibres are polygonal in normal adult muscle while the fibres are round in a child. In infants and children, very little endomysial connective tissue can be seen. In muscular dystrophy, the fibres become rounded and splitting.

3. Necrotic, regenerating and degenerated fibres

When muscle fibres injured, they are stained pale on H&E and infiltrated by phagocytes. It called necrotic fibres with myophagocytosis. It presents in myopathies especially dystrophies like Duchenne muscular dystrophy. Acid phosphatase and esterase reactions highlight necrotic fibres. Necrotic fibres can present in inflammatory myopathies such as polymyositis, dermatomyositis, inclusion body myositis, necrotizing autoimmune myopathies, after rhabdomyolysis and in acute neuropathies. Regenerating fibres are thought to represent an attempt at fibres regeneration, particularly when they appear with vesicular nuclei. Degenerated fibres relate to degeneration rather than regeneration. They appear splitting, pale stained, vacuolated, granular/ragged-red or hypercontracted. On H&E, a coarse granular bluish cytoplasm that represents the ragged red fibres of mitochondrial myopathy on mGT can be present, which called granular fibres. Split fibres appear when a hypertrophic fibre crosses a size limit. However, fibre splitting can be seen at myotendinous insertion in normal. Split fibres represent various myopathies, dystrophies, limb girdle muscular dystrophies and some chronic neuropathies such as Charcot-Marie-Tooth disease. (Figure 2A, B)

4. Atrophic fibres, small and large group atrophy

Atrophic fibres may involve entire fascicle that refer to large group atrophy. Small group atrophy refers to the involvement of atrophic fibres of a small

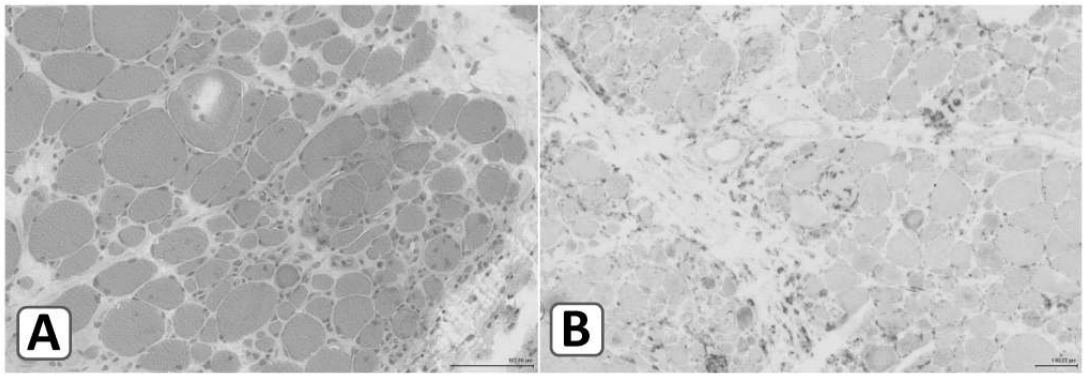

Figure 2 *Necrotic, regenerating, degenerated/split fibres can present in dystrophy, inflammatory myopathies (A) H&EX100 (B) acid phosphatase stainX100*

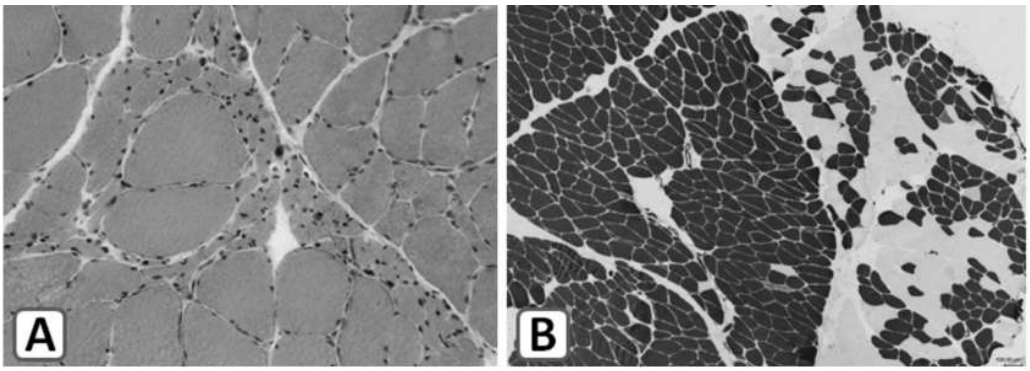

Figure 3 *Large group atrophy, in neurogenic change*
(A) H&EX100
(B) ATPase at pH9.4X40

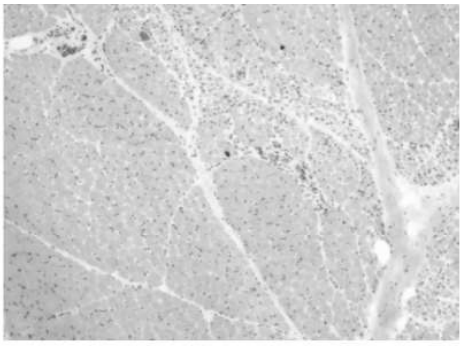

Figure 4 *Perifascicular atrophy, in dermatomyositis H&EX40*

group. Single or several fibres involvement relate to denervation. Sometimes all the fibres type may be atrophic. These patterns are present in neurogenic atrophy. (Figure 3A, B) When the atrophic fibres appear peripherally to a fascicle, it is called perifascicular atrophy that is characteristic finding in dermatomyositis, especially in juvenile type. (Figure 4) Atrophic fibres can present in dystrophies diffusely. Selectively type 1 atrophy can present in congenital myopathies and myotonic dystrophy. Hypertrophic fibres can appear in athletes and as a compensatory change in neurogenic atrophies. They are important findings seen in dystrophy, especially limb girdle muscular dystrophy. Hypertrophy beyond a particular size leads to splitting. Splitting of fibres result in a group of small fibres and may misdiagnose as small group atrophy.

5. Nuclear internalization

Normal muscle nuclei are subsarcolemmal and multi-nuclei. They are small, oval, homogenous and dark staining. However, 3% to 5 % of fibres in a transverse section may show internal nuclei. A Large number of internal nuclei suggests a myopathy and transverse section is best to assess. Dystrophy shows 10-30%, and myotonic dystrophy shows profuse internal nuclei of about 60%. Myotubular/centronuclear myopathy shows more than 30% of single centrally placed nuclei fibres. Charcot-Marie-Tooth disease, chronic neuropathies disease, also shows a large

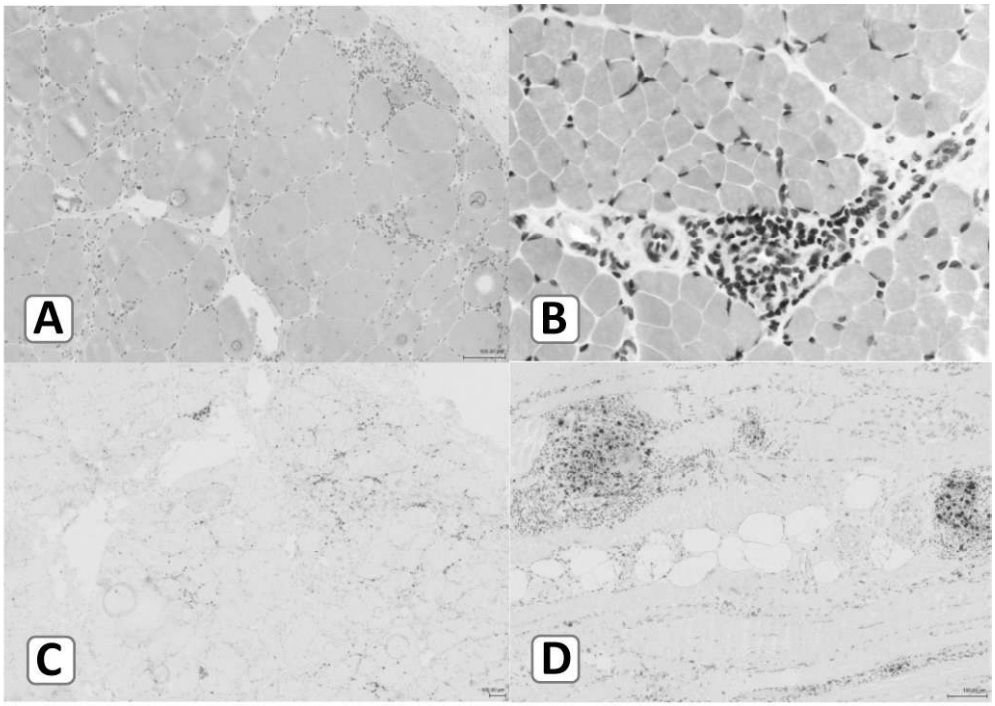

Figure 5 *Cellular reaction (A) endomysial infiltration (B) perivascular infiltration (C) IHC stain for CD8 infiltration (D) IHC stain for CD68 infiltration*

number of central nuclei. Not only increase in number, the character of nuclei may vary in different conditions. Nuclear clump and bag refer to pyknotic, dark staining, clumping chromatin and shrunken nuclei that present in groups. They can appear in neurogenic myopathies and limb girdle muscular dystrophies. Tigroid fibres with granular clumped chromatin are finding in neuropathies and myotonic dystrophy.

6. Cellular reaction, type and distribution of inflammatory cells

Normal muscle shows no any inflammatory cells. Cellular reaction and inflammatory cells infiltrate in inflammatory myopathies like polymyositis, dermatomyositis, and inclusion body myositis. The cellular reaction can present as endomysial or perivascular infiltration. The inflammatory cells mostly consist of B cells, CD4-T cells and dendritic cells in dermatomyositis; CD8-T cells, dendritic cells and macrophages in polymyositis and inclusion body myositis. Immunohistochemical stain can demonstrate these cells. Inflammatory cells also frequently infiltrate in toxic myopathy, necrotizing autoimmune myopathy and dystrophies especially facioscapulohumeral dystrophy (FSHD), Duchenne muscular dystrophy, dysferlinopathy and other limb girdle muscular dystrophies besides inflammatory myopathies. Myophagocytosis, necrotic muscle fibres invaded by mononuclear cells, often present in dystrophies, inflammatory myopathies and some types of limb girdle muscular dystrophies.

7. Endomysial and perimysial connective tissue

In normal muscle, there is very little endomysial connective tissue because each fibre opposes to each other. Endomysial fibrosis or perimysial adipose tissue replacement is pathological finding since it is the change following muscle atrophy and myophagocytosis. Adipose tissue replacement occurs after a period of muscle atrophy or from the chronicity of

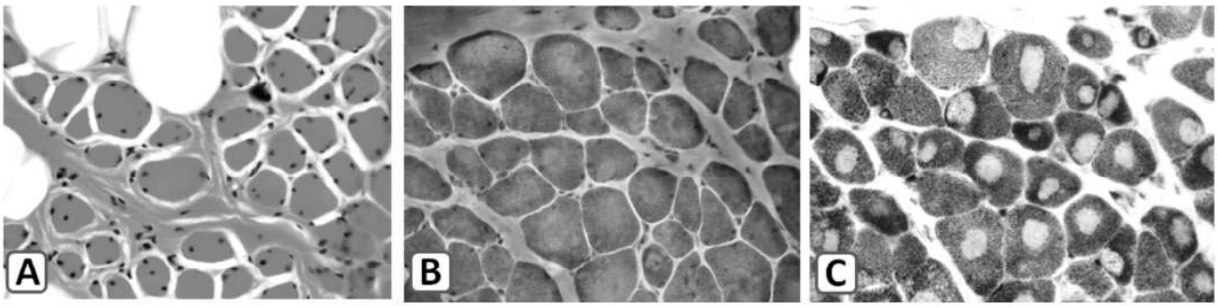

Figure 6 *Cores fibres (A) H&EX100 (B) mGTX100 (C) NADH-TR X100*

the disease. It is the more common change in Duchenne muscular dystrophy but can occur in other dystrophies. However, increased in endomysial connective tissue or fibrosis can occur in some neurogenic myopathies and central core disease (CCD).

8. Changes in fibre architecture and structural abnormalities

Myofibrillar disturbance

Central cores

Central core disease (CCD) is an inherited myopathy caused by recessive *RYR1* mutations characterized by the presence of central cores on muscle biopsy and clinical features of a congenital myopathy.[11] Magee and Shy were the first who recognized the disorder since 1956.[12] Typical central cores fibres present as solitary devoid or uneven stained round abnormal area in the center of the fibre. They usually occupy 30-60% of the cross-sectional area of fibres. They almost present in the central area but may be eccentric. Although a more compact zone of myofibrils seen with mGT stain, they are much more readily identified with oxidative enzyme reactions. The core zone lack of mitochondria and oxidative enzyme activity, in contrast to the normal peripheral zone.[13] The periphery of the core may have enhanced staining, resembling a target or targetoid fibres. Cores fibres may appear with H&E but more easily be identified with mGT and NADH-TR. (Figure 6A, B, C) They are also devoid of other enzymes, such as myophosphorylase, glycogen and ATPase. The cores mostly affect type 1 fibres, and there is often a predominance of type 1 fibres in the biopsy. When the cores appear single and central, they are called central cores, but when they are eccentric and multiple, they have been called multi-mini cores. Engel et al. were the first who report Multiminicores disease (Mmd) in a family with two affected siblings.[14] Although the majority of Multi-minicore disease caused by recessive mutations in the selenoprotein N (*SEPN1*) gene,[15] recessive *RYR1* mutations have been recently identified in distinct subgroups of Multi-minicore disease.[16-18] However, Multi-minicore disease -associated mutations may have clinical features such as external ophthalmoplegia, bulbar involvement and a moderate degree of the respiratory problem that are rare in typical central cores disease. Cores are not limited to central core disease as they can present in the hypertrophic cardiomyopathy associated with missense mutations in the beta-myosin heavy chain gene, *MHY7*[19], autosomal dominant myopathy associated with *ACTA1* gene mutations.[20]

Target fibres

Target fibres look like central cores. They are more focal characterized by three zones. The three

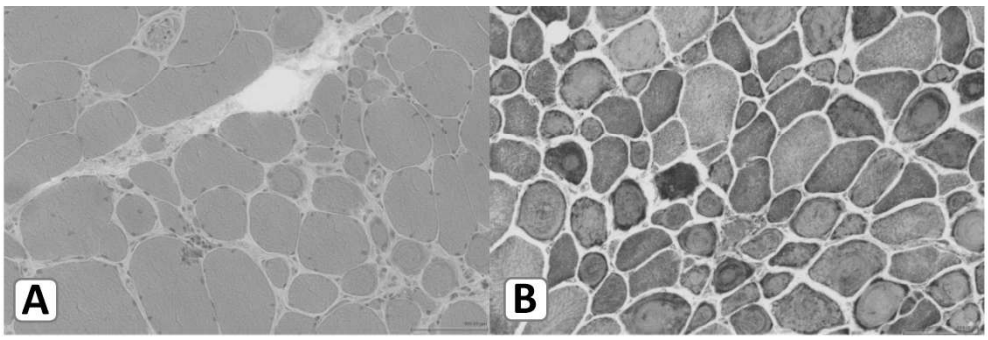

Figure 7 *Ring fibres in Becker muscular dystrophy (A) H&EX100 (B) NADH-TRX100*

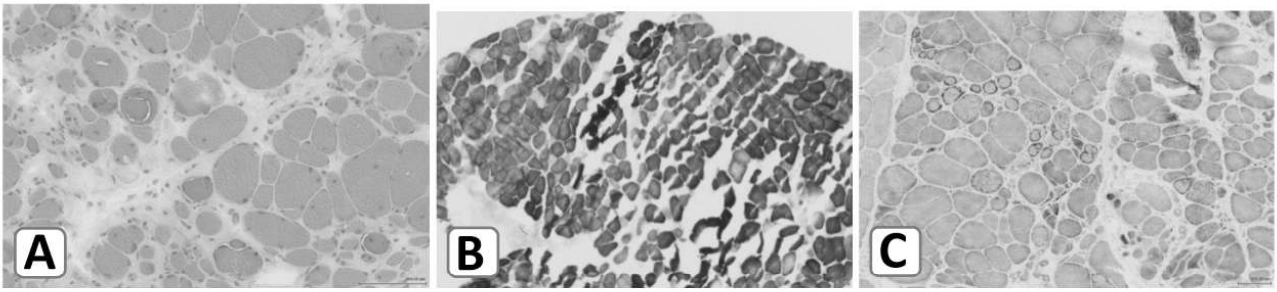

Figure 8 *(A) Whorled fibres H&EX100 (B) Mitochondrial abnormalities appear as ragged blue fibres on COX/SDHX40 (C) Lobulated fibres, NADH-TR X100*

zones consist of; a clear central zone devoid of oxidative enzyme activity; a densely staining intermediate zone with increased oxidative enzyme activity; and a relatively normal peripheral zone of intermediate activity. If the intermediate zone is not clearly present, they are called targetoid fibres. Targets fibres usually associated with denervation process and are most commonly seen in chronic peripheral neuropathies or more acute recovering neuropathies. Experimental studies suggest that they may occur during reinnervation, and they present in association with tenotomy.[21,22]

Ring fibres

Ring fibres appear in several stains, PAS and oxidative enzyme stains. They are present as fibres bounded by a bundle of peripheral myofibrils that radially encircle the internal sarcoplasm. The sarcoplasm is normal in structure and orientation. Rings distinctly appear with PTAH stains, semi-thin sections, or under phase contrast or polarizing microscope. Under EM, the pathologically oriented myofibrils are standard in structure except for hypercontracted sarcomeres. Ring fibres occur in myotonic muscular dystrophy, but they are not pathognomonic of the disease. The significance of ring fibres is still controversial.[3] (Figure 7A, B)

Whorled/coiled/ Snake coils fibres

Whorled/coiled/ Snake coils fibres are characterized by disorientation of longitudinal myofibrils and tend to be more bizarre than the ring fibres. Whorled fibres appear clearly in oxidative enzyme and mGT. They may form giant fibres that seem to be an aggregation of several fibres. It is a non-specific change that can present in many situations including various dystrophies, chronic denervation and other disorders. (Figure 8A)

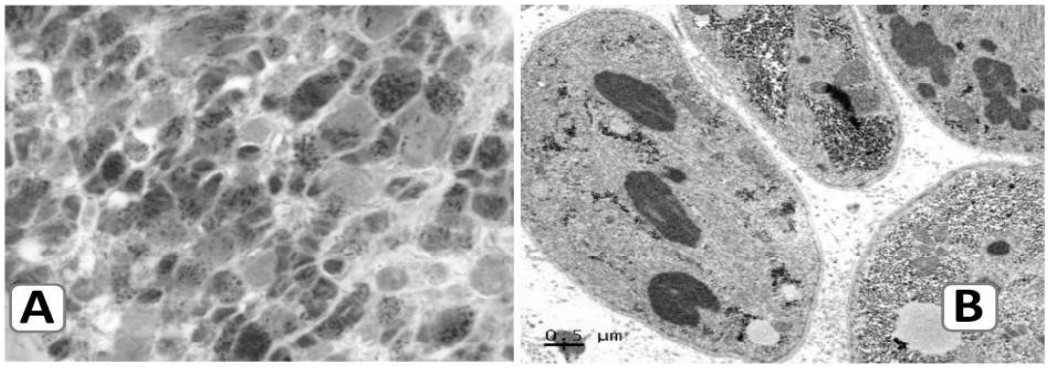

Figure 9 *Congenital NM (A) Rod bodies in subsarcolemmal region, mGTX400 (B) Rod bodies in subsarcolemmal region, EM 10000*

Mitochondrial abnormalities

Mitochondrial abnormalities include the abnormality in structure, number, size and distribution. Even though, the structural abnormality can show with various stains but the ultrastructural microscopy study yield confirmation. They are suspected when the presence of basophilic granularity in individual muscle fibres and an excessively intense reaction with oxidative enzyme stains, or periphery of fibres are intensely particularly stained. Mitochondrial abnormality fibres can found associated with mitochondrial myopathies, but may also be an isolated and incidental finding in occasional fibres in inflammatory myopathies such as polymyositis, inclusion body myositis and dermatomyositis. Mitochondrial abnormalities also occur with the mGT stain by their disruption and "ragged red" appearance. They are dark stained with SDH and NADH-TR. On COX, they show devoid of oxidative reactivity. They appear as ragged blue fibres on COX/SDH stain. (Figure 8B)

Lobulated fibres

Lobulated fibres show intense oxidative enzyme activity with the reactive product at the periphery of the fibre and usually involve type 1 fibres. This area is often triangular composed of many small mitochondria. They are nonspecific and present in many conditions that include limb girdle muscular dystrophies particularly calpainopathy, congenital muscular dystrophy (Ullrich CMD), mitochondrial myopathy and others. (Figure 8C)

Rod bodies

Rods bodies are the hallmark of nemaline myopathies (NM). Shy and colleagues were the first who recognise the disorder since 1963.[23] They proposed the term "nemaline myopathy" derived from "nema", the Greek word, which means thread. Rod or nemaline bodies present as intrasarcoplasmic rod-like structures that are stained red with the mGT preparation. (Figure 9A) They appear as densely dark stained red granules or tiny rods having well demarcated. Although they tend to accumulate beneath the sarcolemma, centrally located rods are not uncommon. Nuclear rods can also be present. They measure about 1-3 mm in width and 3-6 mm in length. On EM, they have a longitudinal striation with a periodicity of 8 nm. (Figure 9B) They have the same structural properties and biochemistry with Z-bodies of the sarcomere. They may arise as a product of polymerization or proliferation of their lattice. On H&E stain, they may appear as faintly defined pink refractile bodies.

They stain negative with ATPase, NADH-TR and SDH as they lack myosin and mitochondria. Multiple gene mutations involving in NM had been reported encompassing a variable clinical spectrum from mild to severe muscle weakness.[24] Mutations in the genes encoding skeletal alpha-actin(*ACTA1*) and nebulin (*NEB*) are the most common. Congenital NM is the most severe form of six clinical presentations according to the classification of Wallgren-Pettersson and colleagues.[25,26] Recently, the author and colleagues had reported a rare case of congenital NM with primary pulmonary lymphangiectasia causing bilateral chylothorax considering that chylothorax being a poor prognostic index and an unusual presentation.[27] However, rods can also be observed in central cores disease, in myotendinous junction, in eye muscle and ageing.[3]

Tubular aggregate

Tubular aggregate (TA) are unusual inclusions within muscle fibres formed from the terminal cisterns of the sarcoplasmic reticulum. [28] They appear as structural change mainly to muscle type 2 fibres, particularly the 2B fibres. They stained strongly with NADH-TR and myoadenylate deaminase (MAD) but are negative for SDH and COX. They stained red with mGT and basophilic with H&E. They are observed in a range of specific neuromuscular conditions in which they are the primary myopathic feature, including familial limb girdle myasthenia, exercise induced myalgia, and gyrate atrophy of the choroid and retina. They also occur in various other disorders in which they are inconsistently present and are often a minor part of the overall myo-pathology. [28] These conditions include periodic paralysis, paramyotonia, and metabolic myopathies, especially ethanol-induced myopathy. Furthermore, they may be features in patients with idiopathic and congenital myopathies. The significant pathology of TA is unknown. Interesting hypothesis that they are a response of the sarcoplasmic reticulum to injuries affecting excitation--contraction coupling or calcium flux had been proposed.[28]

Cytoplasmic bodies

Cytoplasmic bodies are non-specific structural change found within muscle fibres. They can occur in collagen disease and quite common in inclusion body myositis, reducing body myopathy (RBM) and myofibrillar myopathy (MFM). One recognized case with habitual using senna has been reported with this change. They appear as eosinophilic material aggregation with H&E and bright red with mGT. On EM, they are also thought to be an abnormality of the Z line. They show characteristic feature of a dense circular or oval core surrounded by a halo of peripheral fine filaments radiating from it. They usually occur in sporadic fibres but may be extensive throughout the biopsy. They tend to affect selectively type 2 fibres.

Reducing bodies

Reducing bodies appear basophilic with HE and red with mGT. They resemble cytoplasmic bodies but can be distinguished by menadione nitroblue tetrazolium (NBT) method. They appear darkly stained with this technique. They are the characteristic feature of the autosomal dominant, X-linked disorder known as reducing body myopathy (RBM) caused by mutations in the *FHL1* gene.

Vacuoles

Vacuoles can occur in several situations and different types. Conditions which most common are inclusion body myositis, myofibrillar myopathies, distal myopathies, glycogenosis and periodic paralysis. Some vacuoles contain detectable material within,

but some appear as empty spaces. It is paramount to differentiate freezing artefact from this type of vacuoles. Excess lipid droplets may also present as vacuoles with empty spaces. Membrane lined vacuoles associated with two X-linked conditions have been recognized. Kalimo et al. reported one condition characterized by excessive "autophagic vacuoles", which linked to Xq28.[29] Nishino et al. proposed the other caused by *LAMP2* mutations, which also on the X chromosome.[30] Several plasmalemma proteins, extracellular matrix proteins of the sarcolemma and the membrane attack complex localize to these vacuoles having the distinctive ultrastructural appearance. Indentation of sarcolemma also show sarcolemmal proteins, and when sectioned transversely they may have vacuoles appearance. Vacuoles may contain basophilic granular material that appear as red granules with mGT. This type of rimmed vacuole is typical of inclusion body myositis and several other disorders including distal myopathies, oculopharyngeal muscular dystrophies (OPMD) and myofibrillar myopathies. In glycogen storage disease, vacuoles appear empty on H&E. The vacuoles are present in severe childhood cases of acid maltase deficiency (Pompe's disease), adulthood (McArdle's disease) and Glycogenosis V.

9. Enzyme histochemistry and deficiency of enzyme

The absence of particular enzyme is essential for diagnosis. In type V glycogenosis (McArdle disease) there is a complete absence of myophosphorylase and the diagnosis is unequivocal. Staining for phosphofructokinase can also show an explicit absence of protein but such cases are rare. Reduction in the enzyme may be difficult to assess histochemically in some cases. These results should be supported by the biochemical study. Myoadenylatedeaminase may show an absence of stain but the significance of the result may question due to a common mutation in the normal population with the absence of the enzyme. Another important condition is the absence of Cytochrome c Oxidase (COX). The presence of COX deficiency in few biopsies should present with no or very low levels of COX. A more common is the absence of stain in a few fibres. These findings occur in some mitochondrial myopathies, inclusion body myositis and ageing. Type 2 fibres can show very faint stain of COX and caution is needed not to interpret these as negative fibres. The combined COX/SDH yields benefit in interpretation.

Enzyme histochemistry

ATPase: In ATPase preincubated at PH 9.4, type 1 fibres are stained pale and dark in type 2 fibres. The results are opposite when stained at PH 4.6 and 4.3. Type 1 fibres are dark, and type 2A and 2B fibres are stained pale with variable intensity. According to the characteristics staining on ATPase, it is used to demonstrate fibre type proportion and distribution. In quadriceps femoris, type 1, 2A and 2B are one-third each. They are in a mosaic pattern. (Figure 1B) Type 1 fibres of more than 55% are impressed to be type 1 predominance and similarly to type 2A and 2B; each of 55% constitutes an excess of that type. Excess Type 2 consist of more than 80%. Predominant Type 1 indicates a myopathy; either dystrophy or congenital myopathy while type 2 predominance indicates the neurogenic cause. Type I predominance is frequently seen in gastrocnemius and deltoid. Caution is needed to interpret biopsies from these sites. ATPase stains are useful not only for type proportion evaluation, but also fibre type grouping, which is the finding of neurogenic change. Congenital myopathies and myotonic dystrophy may show selective type 1

fibre atrophy. Many conditions show particular type 2 atrophy, such as steroid myopathy, disability, mixed connective tissue diseases, pyramidal tract disease and myasthenia gravis. Type 2 atrophy usually involves type 2B or both 2A and 2B. However, only type 2A atrophy is uncommon. Particular fibre hypertrophy is rare. Subtle changes in fibre size should be evaluated by plotting histograms.[3,10]

Oxidative enzyme: SDH, NADH-TR, COX, COX/SDH

These enzyme histochemical stains reveal various structural abnormalities of muscle fibres. Oxidative enzymes are useful in the diagnosis of mitochondrial myopathies. The abnormal fibres of mitochondrial abnormalities are seen as "ragged blue fibres" or "dark fibres" on SDH, NADH-TR and "ragged red fibres" on mGT. COX is a mitochondrial enzyme, and its activity is absent in mitochondrial myopathies. The combined COX/SDH highlights more abnormal fibres in mitochondrial myopathies as fibres devoid of COX but expressing SDH in blue colour.[3,10] NADH-TR also highlights the sarcoplasmic reticulum and oxidative enzyme activity. Structural abnormalities like cores, targets, whorled and lobulated fibres appear distinctly on NADH-TR.

10. Accumulation of glycogen or lipid

The periodic acid-Schiff (PAS) and Oil red O(ORO) demonstrate glycogen and neutral lipid respectively. These stains are useful for metabolic myopathies. EM study reveals a confirmation of lipid droplets excess. (Figure 10A, B, C) Acid phosphatase identifies lysosomal enzymes and hence identifies necrotic fibres. (Described before in section 3. Necrotic, regenerating and degenerated fibres, Figure2B)

11. Accumulation of amyloid

Congo red and crystal violet stains can detect amyloid. On light microscopy, it stains with faint red colour and shows green birefringence with polarized light. With rhodamine or Texas fluorescence, the deposits show enhanced red fluorescence. Amyloid deposits can occur in inclusion body myositis, myofibrillar myopathies and limb girdle muscular dystrophies with defects in dysferlin and anoctamin 5.[31-33] Ageing can show amyloid deposits.

12. Typical artefacts in muscle biopsy

Artefacts in muscle biopsy that result from inappropriate specimen handling, sectioning or staining procedures, can lead to difficulty in interpretation.[3] Initially cautioned and proven questioned pathological findings to be not artefacts is crucial. Fre-

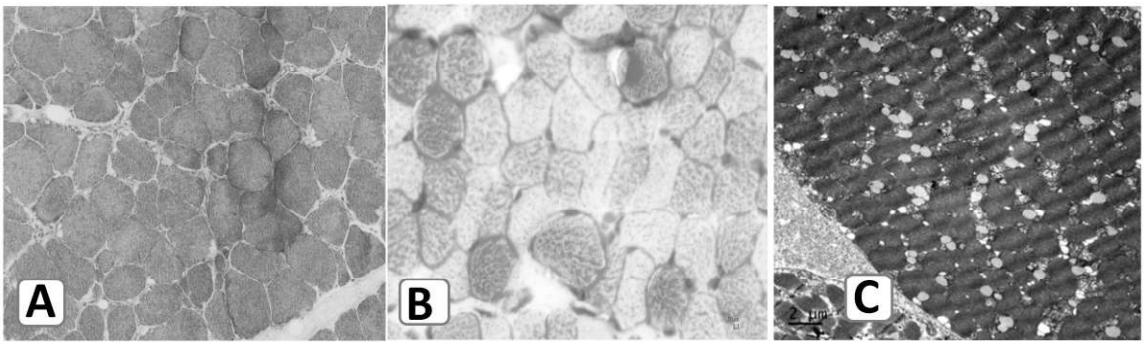

Figure 10 *Metabolic myopathies (A)Vacuolated fibres in glycogenosis, PASX100 (B) Excess lipid droplets in lipid myopathies, OROX100 (C) Confirmation of lipid excess by EM, same case as B, EM 2000*

Table 4 Typical artefacts in muscle biopsy

Artefact	Cause
Ice crystal damage	Too moist before freezing
Holes in fibres	Presence of ice crystal, poor freezing, allowing sample to warm up during transfer, or from the cryostat, or from the freezer
Disrupted or vacuolated fibres	Too moist before freezing
Destroyed fibres at the edge of sample	Rough handling
Rounding up, hypercontracted fibres	Infiltration of local anaesthetic, rough handling
Glycogen depletion	Delayed transportation
Shrinkage, crack fibres and pulling away from endomysium	Poor freezing or too dry sample before freezing
Unevenness of stain	Poor quality staining
Inclusion	Dirt on the specimen section
Striped or ring appearance	Lifting off between the section and slide during staining
Small fibres	Compressed section

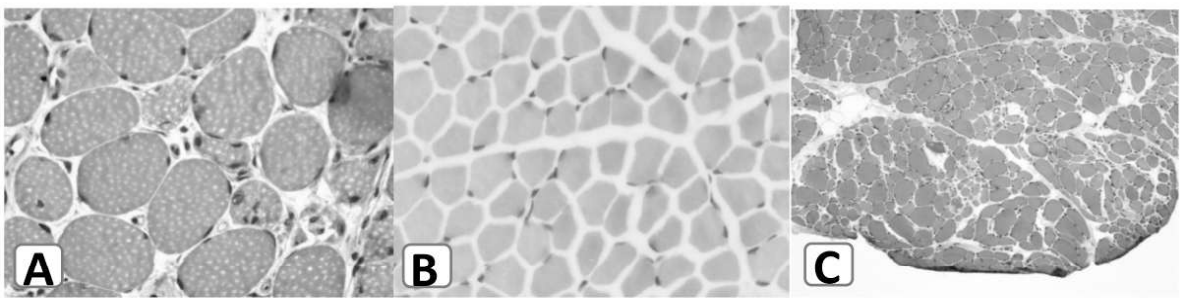

Figure 11 *Typical artefacts (A) Ice crystal damaged (B) Shrinkage, crack fibres pulling away from endomysium (C) Destroyed fibres at the edge of the sample*

quent artefactual results at our laboratory unit are ice crystal damaged, shrinkage, crack fibres and pulling away from endomysium and destroyed fibres at the edge of the sample. (Figure 11A, B, C) Typical artefacts and causes can summarize as Table 4.

Conclusion

Muscle biopsy is a useful tool and approach for diagnostic evaluation of muscle diseases. Ideal utilization of biopsy technique, tissue handling, collecting and transport, good quality staining and pathological findings yield an accurate diagnosis. The authentic interpretation of muscle biopsy correlating with clinical presentation guides appropriate immunohistochemical study and molecular genetic analysis leading to achieve a definite diagnosis.

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Review Article/บทฟื้นฟูวิชาการ

การตรวจทางพยาธิวิทยาของกล้ามเนื้อ (Muscle biopsy)

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ภาควิชาพยาธิวิทยา คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล

บทคัดย่อ

ได้มีการใช้การตรวจทางพยาธิวิทยาของกล้ามเนื้อ(Muscle biopsy) ในการให้การวินิจฉัยโรคของกล้ามเนื้อมานานแล้ว เนื่องจากกลุ่มโรคกล้ามเนื้อมีลักษณะเฉพาะทางคลินิกไม่มากเพียงพอต่อการให้การวินิจฉัยโรคอย่างแน่นอน และโดยเหตุที่มีข้อจำกัดในการตรวจคัดกรองทางพันธุกรรมหรือยีนจำนวนมาก การตรวจทางพยาธิวิทยาของกล้ามเนื้อ(Muscle biopsy) จึงเป็นวิธีที่มีประสิทธิภาพ ประหยัดทั้งเวลาและค่าใช้จ่ายในการให้คำวินิจฉัยโรคของกล้ามเนื้อ จุดมุ่งหมายของบทความฟื้นฟูวิชาการนี้เพื่อเน้นถึงความสำคัญของการตรวจทางพยาธิวิทยาของกล้ามเนื้อ (Muscle biopsy) ผู้เขียนได้อธิบายถึงขั้นตอนของการตรวจชิ้นเนื้อของกล้ามเนื้อทางพยาธิวิทยา การจัดการตัวอย่างชิ้นเนื้อ และอธิบายลักษณะทางพยาธิวิทยาของกล้ามเนื้อจากการตรวจด้วยกล้องจุลทรรศน์ ภายใต้การย้อมสีทางจุลพยาธิวิทยาและการย้อมทางเอนไซม์จุลพยาธิวิทยา โดยแปลผลสัมพันธ์กับข้อมูลทางคลินิก เพื่อผลลัพธ์ในการแปลผลที่ถูกต้องแม่นยำที่สุด

Keywords: Muscle biopsy, Pathology

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