Iraqi JMS

Published by Al-Nahrain College of Medicine
ISSN 1681-6579
Email: Iraqi_jms_alnahrain@yahoo.com
http://www.colmed-nahrain.edu.iq/

Semi-automated Computational Method for Skeletal Muscle Fiber Typing with Lectins: Correlation with Morphometric Studies

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Abstract

Background Muscle fiber typing has been an extensive field of study for many years. Though, limited researches applied lectin histochemistry in the clinical diagnosis of muscle disorders; attention was directed mainly towards enzyme histochemistry.

Objective The use of lectins as recognition systems based on specific protein-carbohydrate interactions in correlation with muscle fibers morphometric standards and optical density features to favor the diagnostic procedures of muscle disorders.

Methods Cross-sections of tibialis anterior muscle from 15 adult rats were stained with Con A, PNA, SBA, WGA, SWGA, LFA, UEA-I, and UEA-II lectins. Photographs of stained sections were analyzed with ImageJ 1.44 software for muscle fiber area, perimeter, optical density, and integrated density.

Results There were statistically significant differences between the parameters of muscle fiber types under study ($P<0.05$) concerning Con A, LFA and UEA-II lectins, but not for the remaining lectins, regarding the optical density and integrated density of muscle fibers.

Conclusions Lectins make accurate recognition of muscle fiber types on fixed paraffin sections when combined with computerized methods to quantify the features seen in muscle biopsies destined for pathological investigations.

Key words Lectins, Muscle fiber typing, Quantitative, Optical density, Morphometry

Introduction

Skeletal muscles are composed of a large number of muscle fibers which differ in their histochemical and physiological properties. Although overlap exists between different fiber types, they can be grouped into Type I fibers (oxidative, slow contracting, and fatigue resistant), Type IIA, or intermediate fibers (oxidative-glycolytic, fast contracting, and fatigue resistant) and Type IIB fibers (glycolytic, fast contracting, and susceptible to fatigue) [(1)]. Muscles, in man, are all of the mixed variety; different types of fibers are not readily distinguished in sections stained by conventional histological techniques, but they are seen quite clearly with the use of more specialized histochemical methods [(2)]. In animals, the histochemical-type fiber designated intermediate resembles, to some extent, human Type IIA fibers [(3)].

Many studies have employed enzyme histochemistry in detecting the metabolic activities of muscle fiber types [(4)]. In addition, some immunocytochemical techniques use fluorescent or enzymatic-labeled antibodies to distinguish different types of fibers; these techniques take the advantage of the ability to probe the myosin composition of fibers in which more than one isoform is present as in human limb muscle with features intermediate between Type IIA and Type IIB [(5)]. Thus, expanding the classification of Type II fibers into more than A

and B subclasses results in the necessity to re-designate human Type IIB fiber as Type IIX [6,7].

Histochemical assessment of the different fiber types in muscle tissue by morphological investigation of muscle biopsy specimens is essential for diagnosis of certain neuromuscular disorders [8]. Determination of muscle fiber types is routinely performed on cryostat-sectioned, unfixed muscle biopsy specimens, using enzyme histochemical reactions; because fixation and embedding destroy this enzyme activity, alternative procedures are preferable for routinely processed specimens [9].

Lectins, on the other hand, have come a long way since their first detection in plants as hemagglutinins to their present status as ubiquitous recognition molecules with myriad exciting functions and applications [10]. Lectins are proteins that bind different carbohydrate motifs. They are important reagents used for studies of changes in the carbohydrate composition of glycoproteins and proteoglycans. Lectins are of non-immune basis; they agglutinate cells and/or precipitate glycoconjugates. These substances bear at least two sugar binding sites. Lectins have no enzymatic activity, may be soluble or membrane bound, and are of bacterial, animal, or plant origin [11]. Such recognition systems based on specific protein-carbohydrate interactions, using carbohydrate-specific tools, can be favorable contributes to diagnostic procedures and to investigations of cell biological processes [9].

There is considerable evidence that lectins are involved in many physiological and pathological events so that cellular protein glycosylation pattern is influenced by several changes, such as the occurrence of disease. Thus, the altered glycoform population of a given glycoprotein may be diagnostic of the disease responsible for the alteration itself [12]. Such abnormal glycosylation has been detected in significant diseases including cancer development in different tissues [13]. For example, the quantitative precipitation method of Con A - carbohydrate interaction was used by Basu and coworkers [14] for differentiation between prostate cancer and benign prostatic hyperplasia, a fact that highlights the growing importance of the use of lectins in the medical field.

These findings are related to the ability of lectins to stain selectively specific structures, especially membranes. In addition, lectins have the capacity to probe specifically the glycoconjugate composition and distribution in cells of the biopsied human muscle, making the application of lectin histochemistry a useful tool in the investigation of muscle disorders [15].

This work aims at investigating the plausibility of using lectin stains for quantitative muscle fiber typing on fixed paraffin sections in correlation with the morphometric standards and optical density features of these fibers. Some clinical implications of such use of lectins in muscle fiber typing are also discussed.

Methods

A sample of 15 adult rats (8 males and 7 females) *Rattus rattus norvegicus albinus* aged 3 months was selected on the basis of being apparently active and healthy, with 300±50 g body weight. Animals were housed 2 per cage and fed standard diet pellets. Cages were 60 cm length by 30 cm width. Animals were anaesthetized with chloroform-impregnated cotton-wool in airtight jars for 2-3 minutes prior to decapitation. Muscle samples were obtained from the freshly sacrificed rats by open excision biopsy. The right and left counterparts of tibialis anterior muscle were chosen in this study for sectioning.

Tissue samples were prepared by fixation with Bouin's solution for 16 hours at room temperature (22 ^{o}C) followed by impregnation in paraffin. This fixation protocol is recommended by Allison [16] in order not to affect the tissue binding sites of lectins. Cross sections of 10 µm thickness were made ready for staining.

Eight types of lectins, Concanavalin A (Con A) from *Canavalia ensiformis* (jack bean) – a mannose binding lectin, peanut agglutinin (PNA) from *Arachis hypogaea* (peanut) and soybean agglutinin (SBA) from *Glycine max* (soya bean) –

galactose binding lectins, wheat germ agglutinin (WGA) and succinylated wheat germ agglutinin (SWGA) from Triticum vulgaris (wheat) – glucose binding lectins, limax flavus agglutinin (LFA) from the slug *Limax flavus* with sialic acid affinity, and Ulex europaeus agglutinin I and II (UEA-I and UEA-II) from *Ulex europaeus* – fucose binding lectins, all from Sigma™ USA were used for staining as follows:

1. Dewaxing the paraffin sections in xylene for 20 minutes then hydrating them through descending concentrations of ethanol alcohol (99%, 90%, 70%, 50%, 30%) each for 3 minutes.
2. Hydrated paraffin sections were washed in normal saline solution (0.9% NaCl) for 10 minutes.
3. In order to keep the slides flooded by the lectin-normal saline solution, the slides were cleaned at the margins of each section by cotton wool to isolate the sections. Spillage of the lectin-normal saline solution was prevented through fitting each slide in a prepared pit on a sheet of softened dental wax.
4. Sections were flooded by lectin-normal saline solution and kept for 90 minutes in a humid chamber, protected from light.
5. Lectin-stained slides were washed in normal saline solution for 20 minutes and mounted in non-fluorescent fractoil (BDH) mountant.

Examination of sections was done with Olympus fluorescent microscope (400X) using a systemic random selection of 5 fields per section. Digital photographs with a camera factor of 3 were taken for the selected fields; these photographs then were transferred to a PC with installed Image 1.44 software. In each field, 10 muscle fibers from each type (I, II, and Intermediate) were identified qualitatively depending on their eye-observed fluorescence intensity [17], and their margins were traced with an optical mouse.

Muscle fibers were segregated into three groups according to their area (area of selection in calibrated square micrometers) and perimeter (length of the outside boundary of the selection in calibrated micrometers) to be given the designation Type I, Type II, and Intermediate, which is in agreement with the previous results of Al-Kaabi work [18].

While in order to measure the optical density of muscle fibers lectin staining, the gray scale level was calibrated according to a standard optical density step tablet [19]. A calibration curve is displayed in figure 1. With tracing of each muscle fiber, the Mean Gray Value within the selection (the sum of the gray values of all the pixels in the selection divided by the number of pixels) was reported in calibrated units (optical density). For red-green-blue (RGB) colored images, the mean was calculated by converting each pixel to gray scale using the formula:

Gray = (Red + Green + Blue) / 3

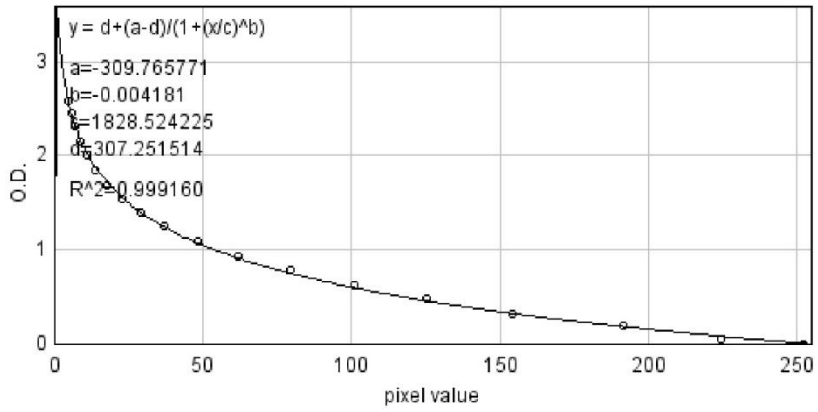

Figure 1. Calibration curve of a standard optical density step tablet. The Mean Gray Value within the selection is displayed in calibrated units representing the optical density of muscle fibers

The calibrated Mean Gray Value of the pixels in the selection was thus calculated, representing the optical density of that muscle fiber, and the Integrated Density (the sum of the values of the pixels in the image or selection, which is equivalent to the product of Area and Mean Gray Value) was measured. Statistical analysis was performed with Microsoft Office Excel® 2010 tool.

Results

The mosaic pattern of muscle fibers was demonstrated with sections stained with Con A, LFA, and UEA-II lectins as shown in figure 2. However, PNA, SBA, WGA, SWGA, and UEA-I lectins did not express apparent muscle fiber typing. Such qualitative typing was more easily observed in sections stained with LFA lectin than those stained with the lectins Con A and UEA-II.

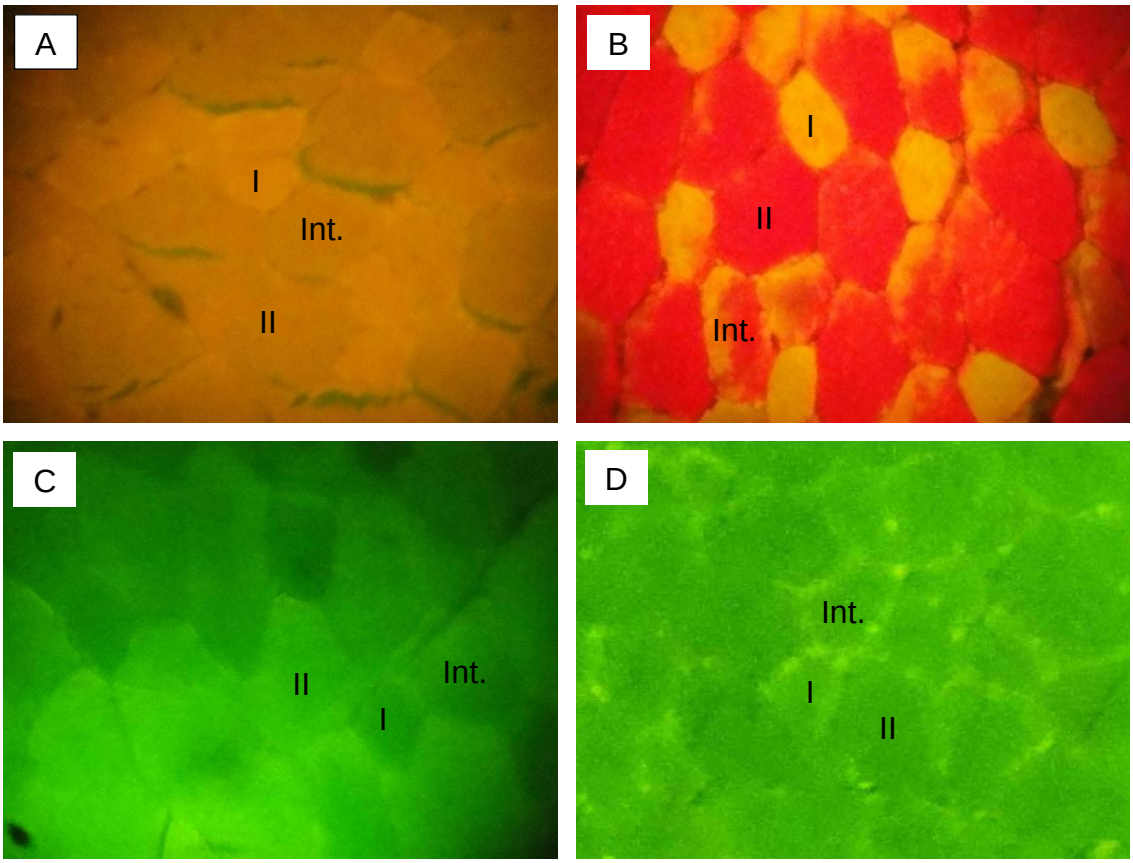

Figure 2. Cross sections of rat tibialis anterior muscle stained with lectins. (A) Con A, (B) LFA, and (C) UEA-II demonstrate fluorescent mosaic pattern of muscle fiber types (I, II, and Intermediate (Int.)). (D) SBA labeled section reveals absent mosaic pattern of muscle fiber types. 400 X. Bar = 50 µm

Table 1 shows the averages of the muscle fiber area, perimeter, optical density, and integrated density for each muscle fiber type stained with Con A, LFA, and UEA-II lectins.

Comparison between the optical density and integrated density of muscle fiber types was done with a single factor ANOVA. The results revealed statistically significant differences between the various muscle fiber types ($P<0.05$) in regard to the studied parameters with Con A, LFA and UEA-II lectins. On the other hand, PNA, SBA, WGA, SWGA, and UEA-I lectins gave statistically insignificant results in relation to the optical density and integrated density of muscle fibers.

Table 1. Averages of muscle fiber area, perimeter, optical density, and integrated density for Type I, Type II and Intermediate muscle fibers stained with Con A, LFA, and UEA-II lectins

Lectin	Fiber Type	Area (μm^2)	Perimeter (μm)	OD[†]	ID[††]
Con A	I	2437.0± 469.6[‡]	199.6± 21.1	97.9± 9.3	239019.4±48339.8
	II	7123.2±1079.8	338.9± 58.9	83.0±11.1	593741.2±141408.2
	Intermediate	3350.6±583.8	228.3±51.5	76.8±20.9	256623.9± 81923
LFA	I	2711.0±251.7	209.6±15.1	122.6±10.9	333457.0±51420.0
	II	7018.7±788.6	332.4±46.5	97.2±8.1	681643.3±89489.9
	Intermediate	3975.5±910.5	255.1±48.3	111.7±5.8	442741.6±95177.0
UEA-II	I	2854.6±514.0	212.4±16.4	40.7±11.6	116973.1±42491.2
	II	8641.7±1598.2	368.1±44.3	65.7±15.6	562837.7±154479.5
	Intermediate	4665.6±1230.6	269.9±47.3	32.9±7.8	156830.6±65460.7

[†] OD = Optical Density, [††] ID = Integrated Density, [‡] ± Standard Deviation

Discussion

In our results, three lectins (Con A, LFA, and UEA-II) showed the mosaic pattern of muscle fibers in concordance with previous works [(17)]. Though these three lectins gave statistically significant difference between the optical density of the three types of muscle fibers, LFA stain was qualitatively better for faster eye recognition of the chess-board appearance of muscle sections as the red-yellow discrimination of colors was easier for the eye to catch, a point in favor of using the computerized methods for the detection of this typing as qualitative examination may be subjective and subtle in the differentiation between these types of muscle fibers. Such quantitative computerized methods have been used for fiber parameters other than the optical density where muscle fiber size was analyzed automatically [(20)].

Five of the lectins used in this study (PNA, SBA, WGA, SWGA, and UEA-I) revealed weak, homogeneously distributed sarcoplasmic reaction. It is interesting that these lectins have different sugar preferences, even though, they failed to stain muscle fibers with different intensities; it could be due to the narrower pattern of sugar agglutination in comparison with Con A, LFA, and UEA-II lectins.

Since complex glycoconjugates are of importance for a number of biological events such as interaction between cells, growth, development, and changes in function of cells, the lectin-staining pattern in skeletal muscle is considered to be very complex; it might be related to development, specialization, and function of the individual muscles [(17)].

Con A has an affinity for cell surface α-D-mannosyl and α-D-glucosyl glycoproteins [(21)], LFA lectin reacts with any sialic acid linkage [(22)], and UEA-II has an affinity for N,N'-diacetylchitobiose [(23)]. In our results, the optical density of Con A stain was more in type I muscle fibers followed by type II then the intermediate, LFA stain optical density was maximum in type I followed by the intermediate then type II, and the optical density of UEA-II stain was highest in type II followed by type I then the intermediate. In other words, mannose, glucose and sialic acid residues were expressed in higher concentrations in type I slow contracting, fatigue resistant muscle fibers, while fucose glycoconjugates were abundant in type II fast contracting, fatigue susceptible fibers. It indicates that muscle fiber types express different levels of glycoconjugates according to their functional specialization, reflected by the different intensities of lectin stains in terms of optical density.

This use of quantitative methods paves the road for the integration of computer programs into more sophisticated analyses regarding glycoconjugate expression detected by lectin

stains; the expanding importance of the ability of lectins to distinguish between delicate variations of oligosaccharide structure makes them perfectly suitable as decoders for such carbohydrate-encoded information. Thus, whilst sugars are able to carry the biological information, lectins are capable of interpreting this "glycocode" [12].

Lectins with binding sites for sialic acid are known to stain the sarcolemma, connective tissues, and blood vessels in both normal and dystrophic muscles, though no difference was reported between the two [8]. Recent methodology, in addition, has characterized a fast, reliable, and inexpensive method of fluorescent lectin staining that can be used for skeletal muscle fiber and general connective tissue visualization during immunofluorescent analysis [11]. This indicates that the sialic acid lectins such as LFA can be used to delineate muscle fiber types with their surrounding connective tissue and capillary network in normal and diseased muscles with the advantage of ease of fiber typing and accurate demarcation of morphometric features.

Latest studies have incorporated different methods in order to reach new approaches for the diagnosis of muscle disorders. These methods include combined immunofluorescence techniques [24] or individualized lectin staining [8]. The use of UEA-II, for example, forms the first model of a legume lectin with an unrestrained binding site and illustrates the importance of hydrophobic interactions in protein-carbohydrate complexes. Together with other known legume lectin crystal structures, it shows how different specificities can be grafted upon a conserved structural framework, so that the different isoforms of myosin may be investigated to yield fiber typing not readily distinguished by currently used histochemical methods [25]. Thus, fucose or chitobiose specific lectins can be now considered under investigation for their significance in muscle fibers, especially during physiological or pathological changes.

In conclusion, lectins are "decoding" the way that how cells are recognizing and interacting with other cells in addition to revealing the exceptional features at the cell membrane and cytosol. The unique glycoconjugates present at the different types of skeletal muscle fibers make the recognition of these types accurate when combined with computerized methods that quantify the percentage of muscle fiber types in larger muscle biopsies destined for pathological investigations. The use of various lectins with overlapping specificity is recommended for staining fixed paraffin sections in order to reach exact identification of muscle fiber types and to make a precise diagnosis when combined with quantitative computerized methods. The role of individual lectins in distinguishing subtle changes in disease conditions of muscle fibers is yet to be studied.

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Received 26th Jan. 2012: accepted 8th Apr. 2012