

An experimental model for the study of collagen fibers in skeletal muscle¹

Modelo experimental para o estudo de fibras de colágeno no músculo esquelético

Eliziane Nitz de Carvalho Calvi^I, Fábio Xerfan Nahas^{II}, Marcus Vinícius Barbosa^{III}, José Augusto Calil^{IV}, Silvia Saiuli Miki Ihara^V, Marcelo de Souza Silva^{VI}, Marcello Fabiano de Franco^{VII}, Lydia Masako Ferreira^{VIII}

^IMaster, Fellow PhD degree, Postgraduate Program in Plastic Surgery, UNIFESP, Sao Paulo-SP, Brazil. Main author. Surgical procedures, data acquisition, manuscript writing.

^{II}PhD, MBA, Associate Professor, Division of Plastic Surgery, UNIFESP, Sao Paulo-SP, Brazil. Conception of the study, manuscript writing, critical revision.

^{III}PhD, Volunteer Faculty, UNIFESP, Sao Paulo-SP, Brazil. Surgical procedures, data acquisition, manuscript writing.

^{IV}PhD, Division of Plastic Surgery, UNIFESP, Sao Paulo-SP, Brazil. Surgical procedures, data acquisition.

^VPhD, Associate Professor, Department of Pathology, UNIFESP, Sao Paulo-SP, Brazil. Interpretation of data, histological analysis, manuscript writing, critical revision.

^{VI}MS, Department of Pathology, UNIFESP, Sao Paulo-SP, Brazil. Interpretation of data, immunohistochemical analysis.

^{VII}PhD, Full Professor, Department of Pathology, UNIFESP, Sao Paulo-SP, Brazil. Interpretation of data, histological analysis, critical revision.

^{VIII}PhD, MBA, Head and Full Professor, Division of Plastic Surgery, UNIFESP, Coordinator Med III CAPES, Sao Paulo-SP, Brazil. Critical revision.

ABSTRACT

PURPOSE: To examine histological and histomorphometric techniques for measuring collagen in skeletal muscle.

METHODS: The following staining methods were used in the study: hematoxylin and eosin, Masson's trichrome, reticulin, and picrosirius red, and immunostaining for collagen types I, II, III, IV, and V. Histomorphometric measurements were performed using Corel PhotoPaint and UTHSCSA Image Tool 3.0 software.

RESULTS: Both the Masson's trichrome and picrosirius red staining provided the best visualization for the measurement of collagen content.

CONCLUSION: This methodology is important for the identification and quantification of the different types of collagen in muscles and can be used in the investigation of the qualitative and quantitative influence of collagen on physical activities, aging, and diseases.

Key words: Collagen. Muscle, Skeletal. Histology. Rectus Abdominis. Cadaver.

RESUMO

OBJETIVO: Examinar os procedimentos empregados na quantificação do colágeno por métodos histológicos e histomorfométricos.

MÉTODOS: Foram utilizadas colorações histológicas por HE, Tricrômico de Masson, Reticulina, Picrosíríus e reação imuno-histoquímica para colágeno I, II, III, IV e V. A quantificação histomorfométrica foi realizada utilizando-se os programas Corel PhotoPaint e Image Tool versão 3.0.

RESULTADOS: Os métodos histológicos de Masson e Picrosíríus apresentaram uma maior facilidade na quantificação do colágeno.

CONCLUSÃO: Este modelo é importante para que possa ser identificado e quantificado os diferentes tipos de colágenos nos músculos e relacionar com a atividade física, envelhecimento e doenças.

Descritores: Colágeno. Músculo Esquelético. Histologia. Reto do Abdome. Cadáver.

Introduction

Extracellular matrix is present in all body tissues and genetic changes in its components can affect many tissues. Collagen fibers are important structural elements of the extracellular matrix¹. Skeletal muscle tissue is composed of two basic types of collagen fibers with different morphological, metabolic, and contractile characteristics². The relative amount and distribution of collagen fibers in the different muscles depend on the species, race, sex, age, muscle group, and on the individual itself. They also depend on the level of physical activity, disuse, nutritional status, denervation, and chronic physiological stress. In this field of research, morphological and histochemical methods of analysis have played an important role in the study of muscle anatomy and physiology¹.

Although a large number of collagen types have been identified, the exact role of their structural functions in the different tissues of the body, especially in muscles, is still not well understood. However, it is known that collagen in striated muscles has a structural function connecting muscle fibers to ensure that these fibers are properly aligned. The tensile strength of collagen results from the unique structure of its fibers, fibrils, and molecules. More specifically, the tensile strength results from inter- and intramolecular crossed connections, orientation, density, and frictional forces between fibers, and physical and chemical interactions with other structural components of the extracellular matrix³.

The evaluation of collagen fibers and particularly their subtypes has been essential for the diagnosis of different pathologies⁴ in muscle biopsies and experimental conditions, including hypertrophy caused by repetitive strain and sustained work, muscle atrophy due to lack of use or denervation, and longitudinal fiber splitting¹. Muscle collagen has multiple mechanical, immune, and tissue repair functions. It also plays an important part in the transmission of forces resulting from active muscle contraction^{3,5}. Collagen is an important component of muscles and fasciae that provides strength to the structures⁶⁻⁹. At present, more than 19 types of collagen have been described¹⁰, with the types I, II and III being the most studied ones. Collagen types I, III, IV and V are found in striated muscles and only types I and III are found in the fascia⁹.

Type I collagen is the most common and accounts for 90% of the total collagen in mammals. It is synthesized by fibroblasts, odontoblasts, and osteoblasts. It is composed of two alpha-1 and one alpha-2 chains and usually organized into thick bundles, which confer resistance to structures. Type-1 collagen is found in

ligaments, tendons, and fasciae^{11,12}.

Type III collagen is composed of three alpha-1 chains and forms shorter and thinner fibers. It is synthesized by fibroblasts and reticular cells, and generally found associated with type I collagen in different ratios. Type III collagen is prevalent in tissues with some degree of elasticity, including the skin, muscles, fasciae, and ligaments¹².

Type IV collagen is the most abundant structural component of the basement membrane, providing it with mechanical strength. It consists of collagen molecules, which are not assembled into fibrils, but which are attached end-to-end to form a network structure. Type IV collagen is found associated with various non-fibrous components of the extracellular matrix forming a continuous membrane separating some tissues. It may also serve as a filtering unit as evidenced by its function in the kidneys, where the blood is filtered. Type IV collagen is produced by epithelial cells, muscle cells, and endothelial cells of blood capillaries¹².

Type V collagen regulates the diameter of collagen fibers. It has the lowest relative mass among the collagen types, but plays an important role in cell proliferation and tissue repair. The presence of type V collagen in the basement membrane of vessels and in certain mesenchymal tissues is of fundamental importance for the binding of basement membrane type IV collagen to the loose connective tissue of organs. It also actively participates in the interaction of extracellular matrix components associating with other types of collagen, including collagen types I and III¹³.

Thus, it is clear that the collagen family has multiple functions and that collagen molecules are arranged in different configurations according to their function. There is strong evidence that collagen molecules have been modified during the evolution of multicellular organisms and that they have gradually adapted to the several functions that were created in that process.

Therefore, it is important to evaluate the collagen composition of the different muscles, investigate changes in the relative amount and distribution of collagen fibers in the skeletal muscle with age, and the effect of physical exercise on collagen synthesis.

The purpose of this study is to describe histological and histomorphometric methods for assessing collagen fibers in skeletal muscle.

Methods

The study was approved by the Research Ethics Committee of UNIFESP (approval number CEP 1492/08) and

authorized by the Institute of Legal Medicine of Franca (SP, Brazil). Fresh adult male cadavers, at room temperature, were dissected within 24 hours of death. Exclusion criteria comprised cadavers younger than 18 years of age, stored at low temperatures, with previous laparotomy, abdominal wall hernias, or abdominal trauma.

Tissue samples with an area of 1 cm² were collected from the rectus abdominis muscle 3 cm above and 2 cm below the umbilicus (Figure 1), and labeled in ascending numerical order, so that observers remained blinded to the age of the subjects during histological analysis. The specimens were then fixed in 10% buffered formalin for 24 hours, dehydrated in ascending concentrations of ethanol up to absolute ethanol, cleared in xylene, and embedded in paraffin blocks. Next, specimens embedded in paraffin were cut into 4- μ m sections using a microtome. The histological sections were mounted onto slides and stained with hematoxylin and eosin (H&E) and special stains for collagen, including picosirius red, Masson's trichrome, and reticulin, and immunostained with antibodies for collagen types I, II, III, IV, and V.

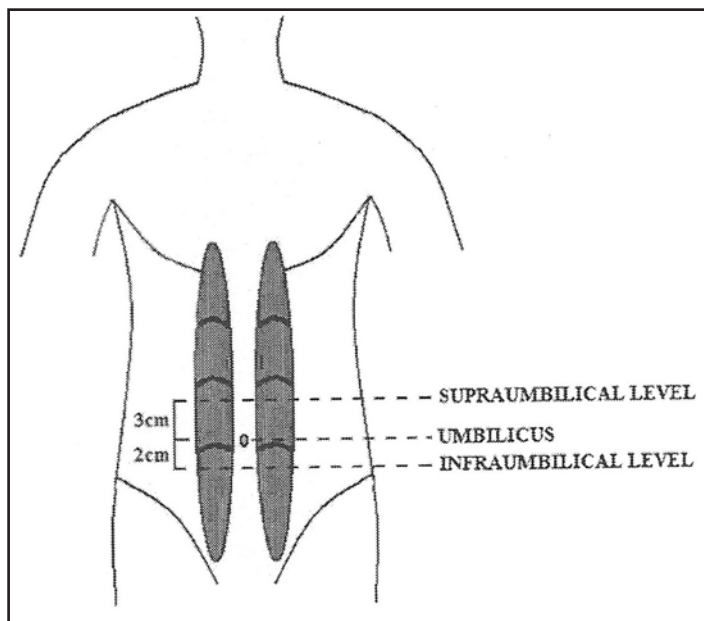


FIGURE 1 – Sites from which fragments of the rectus abdominis muscle were collected for analysis.

Hematoxylin and eosin staining

Routine histological staining with H&E was performed. The histological sections were deparaffinized in xylene, rehydrated through a graded series of ethanol, and washed in running water. The sections were then immersed in Harris' hematoxylin for two minutes, washed in running water (five minutes), rinsed in distilled water (one minute), stained in an aqueous solution of eosin (five

minutes), and dehydrated in ascending concentrations of ethanol. Next, the sections were cleared in xylene (three successive changes, one minute each) and mounted under a cover-slip in Entellan¹⁴. The histological sections were stained with H&E for the detection of technical artifacts and histopathological changes (Figure 2). Sections containing these features were excluded from the study and were not analyzed quantitatively.

Picosirius red staining – Quantitative analysis of types I + III collagen

First, the histological sections were deparaffinized in xylene (three changes, one minute each), rehydrated through a graded series of ethanol (100%, 90%, and 70%, one minute each), and rinsed in water. The sections were then immersed in saturated aqueous picric acid (20 minutes), stained with a 0.1% Sirius Red (F3BA) solution in saturated aqueous picric acid (60 minutes), and rinsed in distilled water¹⁴. Following, the sections were dehydrated, cleared, and mounted under a cover-slip. The slides were examined by polarized light microscopy for the quantification of collagen fiber content. Samples obtained from all different sites were stained with picosirius red (Figure 2) and examined by polarized light microscopy (Figure 3) for the quantification of collagen types II and III¹⁵, without differentiating between them.

Masson's trichrome staining – Quantitative analysis of type I collagen

Tissue samples obtained from all different sites were stained with Masson's trichrome for the quantification of type I collagen. The histological sections were deparaffinized, rehydrated, washed in running water (two minutes), immersed in 5% iron alum (ten minutes) and Regaud's hematoxylin (three minutes), and rinsed in distilled water, 95% alcohol, and picric alcohol. Then, the sections were washed again in running water (ten minutes), rapidly immersed in xylydine ponceau aqueous solution (three minutes), distilled water and 1% glacial acetic acid, and 1% phosphomolybdic acid (three minutes). Next, they were rinsed again in distilled water, immersed in aniline blue for 2-5 minutes, dehydrated, cleared, and mounted under a cover-slip (Figure 2)¹⁶.

Reticulin staining – Quantitative analysis of type III collagen

This procedure was used for the quantification of type III collagen. The histological sections were deparaffinized, rehydrated, rinsed in distilled water, and immersed in 5% potassium permanganate (two minutes). The sections were rinsed in the

following order: distilled water, 5% oxalic acid, distilled water, 2% iron alum, distilled water (six minutes), Gomori's solution (90 seconds), distilled water, 10% formalin (two minutes), gold chloride (1:500) for two minutes, and 5% sodium thiosulfate. Then the sections were dehydrated through a graded series of ethanol, cleared in xylene, and mounted under a cover-slip (Figure 2).

Immunohistochemistry

Sections 3µm in thickness were deparaffinized in three changes of xylene, rehydrated in a graded series of ethanol, and rinsed in distilled water. For antigen retrieval, the slides were immersed in 0.5M acetic acid for 90 minutes in water bath at 37°C. Endogenous peroxidases were quenched with 3% hydrogen peroxide for 20 minutes at room temperature. Sections were incubated overnight at 4°C with the following primary antibodies diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA): purified rabbit anti-human type I collagen (Novotec, ref. 20111) at a dilution of 1:50; purified rabbit anti-human type II collagen (Novotec, ref. 20211) at a dilution of 1:100; purified rabbit anti-human type III collagen (Novotec, ref. 20311) at a dilution of 1:50; and purified rabbit anti-human type IV collagen (Novotec, ref. 20411) at a dilution of 1:100. After incubation, the sections were washed in PBS, incubated with biotinylated secondary antibody (LSAB, Dakocytomation) for 30 minutes, washed in PBS, and incubated with streptavidin-peroxidase conjugate (LSAB, Dakocytomation) for 30 minutes. Next, the reaction was developed using 3,3'-diaminobenzidine tetrahydrochloride (Sigma) for five minutes. Sections were briefly counterstained in hematoxylin, dehydrated, and mounted under a cover-slip (Figure 3). Negative and positive controls were run simultaneously.

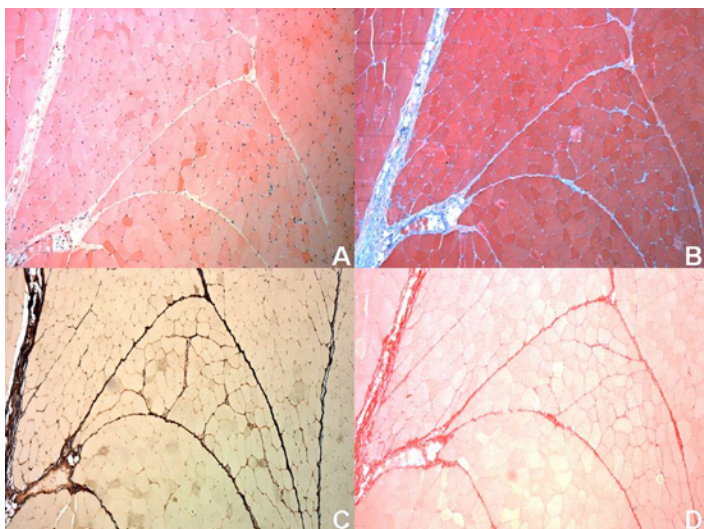


FIGURE 2 – Micrographs of histological sections of the rectus abdominis muscle stained with (A) H&E; (B) Masson's trichrome; (C) reticulin; and (D) picrosirius red. Magnification 100x.

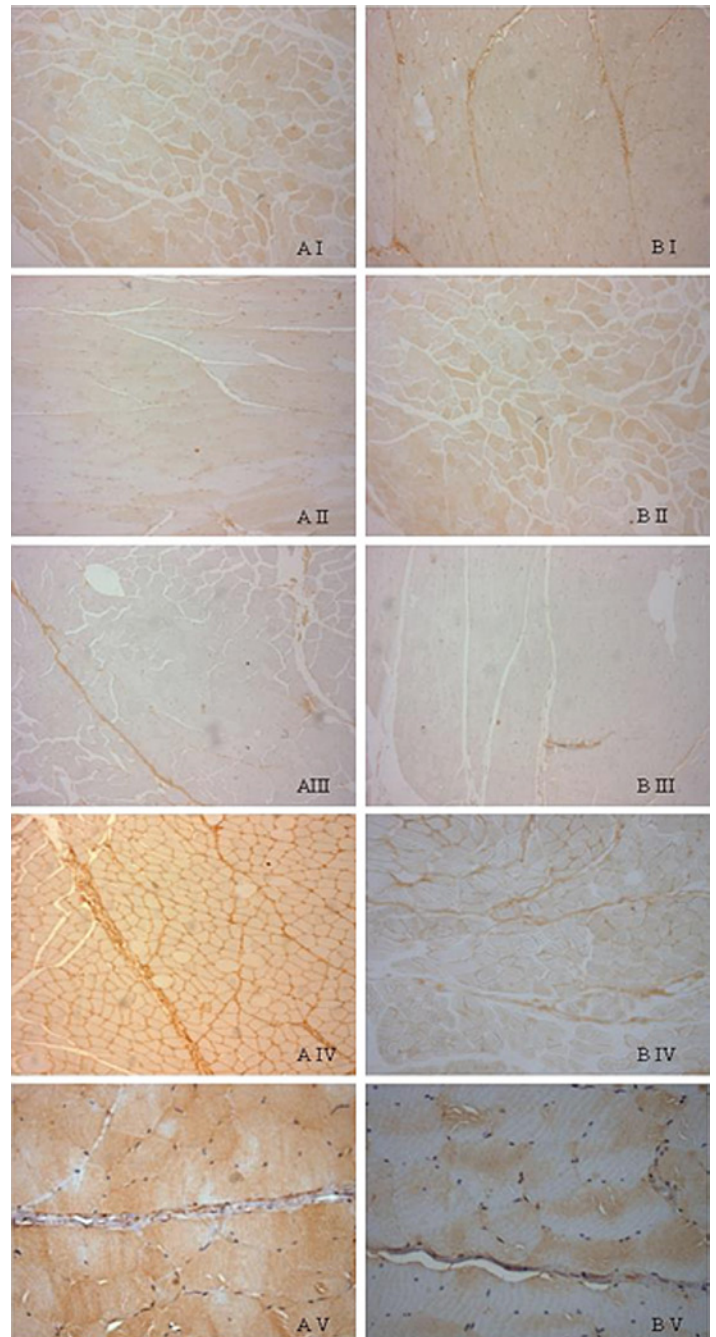


FIGURE 3 – Micrographs showing (AI, BI) type I collagen; (AII, BII) type II collagen; (AIII, BIII) type III collagen; (AIV, BIV) type IV collagen; and (AV, BV) type V collagen detected by immunohistochemistry in the rectus abdominis muscle. Magnification 100x.

Histomorphometric analysis

Image acquisition and digitization with 200 dpi resolution was performed using a 3.2 megapixel digital camera (Olympus Q-Color3 Imaging System, USA) coupled to an optical microscope (Olympus BX40). Images were acquired at 100x magnification from ten randomly chosen fields. The objective lens, microscope brightness, and height of the condenser, as well as the saturation, brightness, and contrast settings of the image acquisition software

were standardized to ensure uniformity of the digitized images. Images from slides stained with Masson's trichrome and reticulin (Figure 4) were transferred to a personal computer and processed with Corel PhotoPaint 14 (Corel Corporation, Mountain View, CA, USA), which was also used to generate masks to separate areas with specific colors.

A specific structure can be separated from the whole image using the color mask function and by selecting the most frequently occurring pixel color in this structure; then the software automatically selects adjacent pixels of similar color. The selected objects can be protected by inverting the mask using the command 'invert' and the rest of the image can be removed with the tool 'eraser'. This allows an easy selection of a large number of objects in an efficient and precise manner¹⁷. The processed images showing the selected collagen fibers were then analyzed using the UTHSCSA Image Tool 3.0 software (University of Texas Health Science Center, San Antonio, TX, USA) for morphometric measurements and determination of the relative amount of collagen fibers given by the ratio of the area containing collagen fibers to the total area of the image expressed in μm^2 . The slides stained with picrosirius red were examined by polarized light microscopy (Figure 4) to assess the birefringence of collagen fibers, which appear in bright green, yellow, or orange colors. Images taken under polarized light were digitized and collagen fibers showing birefringence were counted using the Image Tool software (Figure 4).

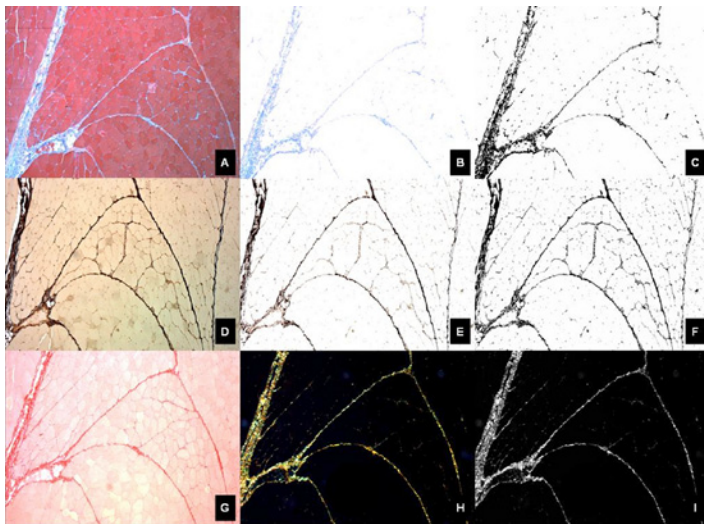


FIGURE 4 – Micrographs of histological sections of the rectus abdominis muscle stained with (A-C) Masson's trichrome; (D-F) reticulin; and (G-H) picrosirius red. Fields selected in (B, E) Corel PhotoPaint, (H) examined by polarized light microscopy, and (C, F, I) exported to the Image Tool software for quantification of collagen fibers. Magnification 100x.

Discussion

Image analysis has been used in pathology for accurate and precise measurements and counting of different features. Important information obtained by morphometric analysis of tissue and cellular components can be used for diagnostic and prognostic purposes¹⁷. The use of quantitative morphometric analysis as an effective and low cost tool has been discussed in the literature for more than 30 years. However, its use has been limited to a small number of diagnostic and research centers. One possible reason for this may be the fact that there is little evidence in the literature that this technique can provide stable, specific, selective, and reproducible algorithms for the diagnosis, prognosis, and evaluation of pathologies¹⁷.

The human eye is sensitive to a number of factors, including luminosity and variation in contrast and brightness and therefore any visual examination is open to subjective interpretation. The objective of morphometry is to eliminate subjectivity and increase the reproducibility of measurements¹⁷.

Immunocytochemistry can be employed to identify the different types of collagen, but in addition to the high cost associated with this procedure, it is a more complex technique. Routine histological techniques can also be utilized for the qualitative and quantitative assessment of collagen content. H&E staining can be used to assess tissue viability; picrosirius red staining combined with polarized light microscopy is the best technique for visualization of collagen types I and III; Masson's trichrome staining can be used to assess type I collagen; and reticulin staining to assess type III collagen.

Each type of collagen has a specific function in muscles and their amount may decrease with age. Certain physical activities and exercises may change collagen composition. The quantification of the relative proportion of collagen types in muscles may help physical therapists and physicians to determine the most appropriate therapy that may contribute to the repair of muscle lesions.

Conclusion

The methodology described in this study is important for the identification and quantification of the different types of collagen in muscles and can be used in the investigation of the qualitative and quantitative influence of collagen on physical activities, aging, and muscular diseases.

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Correspondence:

Eliziane Nitz de Carvalho Calvi
Disciplina de Cirurgia Plástica, UNIFESP
Rua Napoleão de Barros, 715/4º andar
04024-0022 São Paulo – SP Brasil
fisiobeliziane@yahoo.com.br

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