Stereological Quantification of Extraocular Muscles in Humans

Cuantificación Estereológica de Músculos Extraoculares en Humanos

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SUMMARY: The aim of this study is to quantify muscular and connective tissue volumes of extraocular muscles (EOM) in humans with no ophthalmological disease using stereology. EOM from five cadaveric non-strabismic humans were obtained. The number of muscle fibers in 5,000 μm^2 and volume density (Vv) of muscle and collagen were measured using stereology. Comparisons between antagonist EOM were conducted using Wilcoxon signed rank test for paired samples. A secondary analysis examining differences between pairs of EOM was also conducted. Bilateral tests were performed, and significance was set at 0.05. The horizontal rectus muscles (medial and lateral rectus) had the highest Vv of muscle and the lowest Vv of collagen. The inferior rectus muscle tended to have a fewer number of fibers per 5,000 μm^2 than the rest of the EOM. However, these differences did not reach statistical significance. This is the first published study describing the normal histology of human EOM using stereology. Our investigation, through the quantification of the proportion of muscle and collagen tissue, as well as the number of muscle fibers in 5,000 μ^2, establishes normal stereological parameters for EOM of humans without ophthalmological disease.

KEY WORDS: Oculomotor muscles; Humans; Histology; Anatomy.

INTRODUCTION

The oculomotor system has the role of providing a stable image in the retina and allows searching visual targets in space, with both rapid-saccadic and slow-pursuit movements. The ocular alignment and a steady fixation are key factors in order to achieve high grade visual acuity and stereopsis. The extreme precision of the ocular movements relies not only on the complex supranuclear and nuclear neural control, but also on the complex biological organization of the extraocular muscles (EOM) (Porter *et al*., 1995; Spencer & Porter, 2006).

The EOM are a group of seven skeletal muscles: medial rectus (MR), lateral rectus (LR), inferior rectus (IR), superior rectus (SR), inferior oblique (IO), superior oblique (SO) and levator palpebrae (LP). These muscles show numerous differences when compared to other skeletal muscles, such as masticatory or limbs muscles. For this reason, they are actually defined as a different allotype (Hoh *et al*., 1989). In contrast to limbs muscles, EOM are divided in two main compartments, an orbital, more external compartment which inserts in the orbital pulleys, approximately at globe equator, and a global, more internal compartment that reaches the sclera (Büttner-Ennever, 2007). In relation to motor units (MU), the EOM MU sizes are one order of magnitude smaller than those in limbs muscles, and also have about 4 times higher firing frequencies, and faster contractile properties (Yu Wai Man *et al*., 2005). These characteristics allow higher precision and velocity in movements. EOM fibers express all known heavy-chain myosin isotypes (Davis *et al*., 1986), while limbs muscles express only 4 of them (Briggs & Schachat, 2002; Schachat & Briggs, 2002). This in part explains why the contractile properties of both muscle groups are not similar. EOM have also higher mitochondrial content, metabolic rate and a more developed microvascular network, features that make them particularly fatigue resistant (Wooten & Reis, 1972; Fuchs & Binder, 1983; Carry *et al*., 1986). EOM fibers do not fit in the classical 4-type classification of skeletal muscle fibers. They are actually divided in 6 types of EOM fibers, regarding its location (orbital/global), color (white, intermediate or red) and innervation patterns (single or multiple). The multiple innervated fibers, also known as tonic-type fibers, are exclusively present in EOM. These fibers are smaller, located more superficially in the muscle, contract slower, and they are involved in slow pursuit movements.

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The single innervated fibers, or twitch-type fibers, are seen in all types of skeletal muscle. They are bigger, located deeper in the muscle and involved in rapid-saccadic movements. EOM have even a distinct immune response, having less capacity to control complement reaction (Yu Wai Man *et al*.; Spencer & Porter). All these differences make EOM prone to certain diseases, being selectively spared in Duchenne muscular dystrophy and targeted in thyroid eye disease or ocular myasthenia gravis.

The EOM are particularly relevant in strabismus, being the main therapeutic target in procedures that modify their function (i.e. botulinum toxin injection) and anatomy (i.e. strabismus surgery) in order to correct ocular misalignment (Davis *et al*.; McNeer *et al*., 2000). EOM histology also undergoes changes in strabismic individuals. Semi-quantitative studies have described certain changes in EOM samples of humans with horizontal or vertical strabismus, showing an increased amount of elastin and collagen, and an augmented expression of connective tissue related genes compared to EOM of non-strabismic human controls (Stager Jr. *et al*., 2013; Agarwal *et al*., 2016). Nevertheless, histopathology of EOM in strabismus is far to be fully understood (Schiavi, 2016). In fact, there are no histological studies published that quantitatively describe muscle volume, and connective and vascular tissue of EOM, in both healthy and strabismic humans.

The stereological method quantitatively analyses biological tissue samples, giving as result numerical parameters that are unbiased, accurate and reproducible, allowing an easier comparison between different groups (Mandarim-de-Lacerda & del Sol, 2017).

The aim of this study is to estimate muscular and connective tissue volumes of EOM in humans with no ophthalmological disease using stereology.

MATERIAL AND METHOD

The specimens studied were five cadaveric human orbits from five male adults without known ophthalmic disease. The samples were donated to the Pontificia Universidad Católica de Chile, and the study was conducted with prior approval of the ethic committee of our institution (project number 12-074).

The orbits were dissected within the first 24 hours post-mortem and were preserved in cold between 0-4º Celsius. The orbital content was removed in toto within the periorbita, through the superior and medial orbital walls. Four rectus

muscles and two oblique muscles were isolated from each orbit. The muscles were fixated in a tamponade solution of formaldehyde 10 % (pH = 7.4) for 10 days and then embedded in paraffin. Following the stereological procedure described by Mandarim-de-Lacerda & delSol (Mandarim-de-Lacerda & del Sol, 2017) multiple sections of 5-μm thickness were taken. The sections were stained, obtaining five hematoxylin-eosin, five periodic acid-Schiff (PAS) and five Van Gieson stained sections from each EOM. Five different random fields were evaluated from each stained section. So finally, for each muscle group (for example medial rectus muscles), we analyzed 125 hematoxylin-eosin fields (estimate number of muscle fibers), 125 PAS fields (estimate volume density of vessels) and 125 Van Gieson (estimate volume density of muscle and connective tissue).

For the stereological analysis, every field was digitized using a Nikon® microscope, model Eclise 80-i, with 4x, 20x and 40x magnification. Volume Density (Vv) is defined as the volume of a component (in this case muscle, vessels or connective tissue) per unit volume of the containing reference space (the field analyzed) and is expressed as a percentage. In relation to data analysis, pairwise comparisons of Vv Muscle, Vv Collagen and number of fibers per 5,000 μm^2 between antagonist EOM were conducted using Wilcoxon signed rank test for paired samples. A secondary analysis examining differences between pairs of EOM (vertical, horizontal and oblique) was also conducted. Significance values were based on two-tailed tests, and p-values <0.05 were considered statistically significant. No correction by multiple comparisons was performed in this study.

RESULTS

The mean and standard deviation (SD) of muscle Vv, collagen Vv and number of fibers per 5,000 μm^2 by extraocular muscle are detailed in Table I.

Regarding the Vv of muscle, pairwise comparisons showed no statistically significant difference between EOM groups (p-value > 0.05). However, the Vv of muscle of the medial and lateral rectus tended to be higher when compared with the rest of the EOM (Fig. 1). This was more evident when comparing each horizontal muscle, with the superior and inferior rectus, and the superior oblique (p-value 0.06 each pairwise comparison).

In terms of Vv of collagen, the lateral rectus had the lowest mean of 26.6 followed by the medial rectus with a mean of 28.4 (Table I). The vertical (superior and inferior) rectus and superior oblique muscles showed a similar mean

Table I. Summary of characteristics by extraocular muscle.

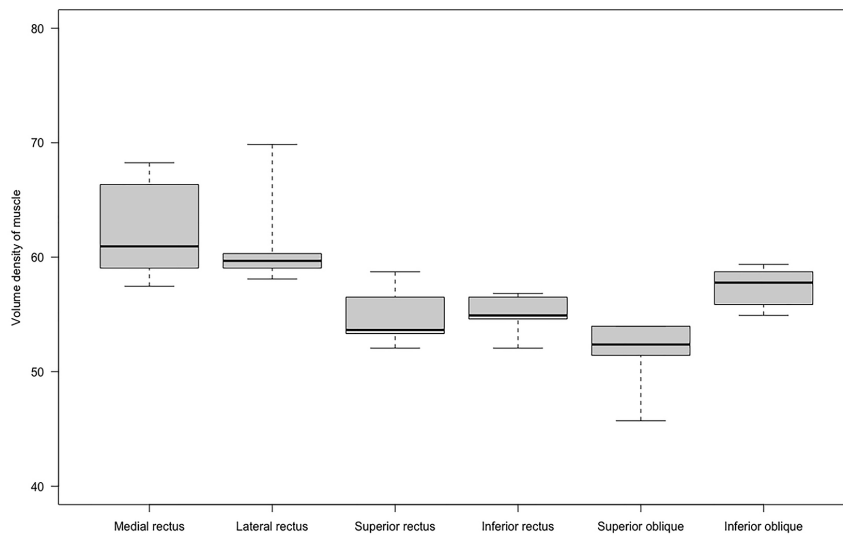| Muscle	Number of samples	Volume density of muscle	Volume density of collagen	Number of fibers in 5,000um^2
		Mean ± SD		
Superior rectus	5	54.9 ± 2.7	32.3 ± 3.1	17.4 ± 1.3
Inferior rectus	5	55.0 ± 1.9	32.4 ± 3.0	16.8 ± 0.9
Medial rectus	5	62.4 ± 4.7	28.4 ± 3.0	18.4 ± 3.5
Lateral rectus	5	61.4 ± 4.8	26.6 ± 3.8	18.8 ± 2.0
Superior oblique	5	51.5 ± 3.4	32.6 ± 2.4	18.2 ± 0.8
Inferior oblique	5	57.3 ± 1.9	30.8 ± 3.4	19.4 ± 2.2

Fig. 1. Boxplot of volume density of muscle by extraocular muscle. The medial and lateral rectus tended to have higher volume density of muscle. There was no statistically significant difference between extraocular muscles (p-value > 0.05).

of Vv of collagen, higher than the horizontal rectus. However, when comparing each horizontal rectus with the superior rectus (p-value 0.1), the inferior rectus (p-value 0.06), and the superior oblique (p-value 0.06) muscles, this difference was not statistically significant. The Vv of collagen by extraocular muscle is shown in Figure 2.

Concerning the number of muscle fibers per 5,000 µm^2, greater dispersion was seen in the horizontal rectus and inferior oblique muscles compared to the vertical rectus and superior oblique (Fig. 3). No statistical differences in pairwise comparison (p-value > 0.05) were found. However, the inferior rectus tended to have a fewer number of fibers than the rest of the EOM. The number of fibers per 5,000 µm^2 by extraocular muscle is shown in Figure 3.

Examples of Van Gieson (collagen and muscle volume densities) and Hematoxylin-Eosin (number of fibers per 5,000 µm^2) stains, for each muscle group, are shown in Figures 4 and 5.
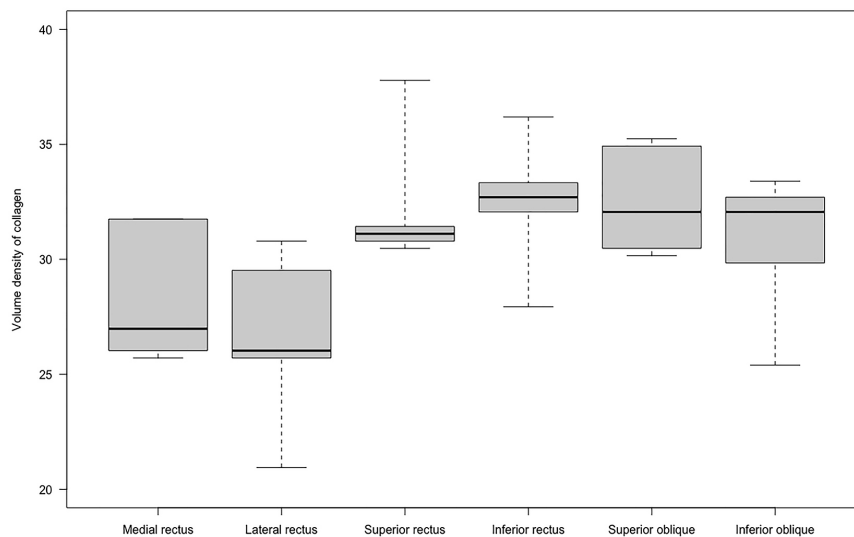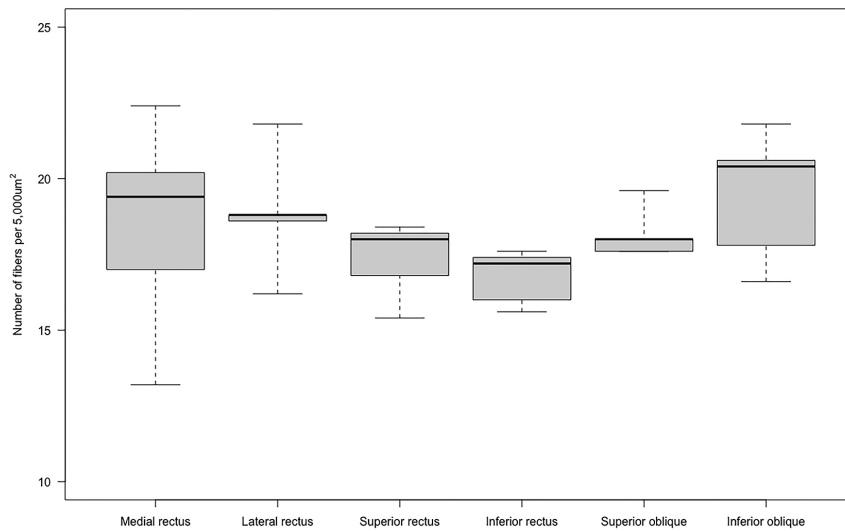

Fig. 2. Boxplot of volume density of collagen by extraocular muscle. The medial and lateral rectus tended to have lower volume density of collagen. There was no statistically significant difference between extraocular muscles (p-value > 0.05).

Fig. 3. Boxplot of number of muscular fibers per 5,000 μm^2 by extraocular muscle. The horizontal rectus and inferior oblique had greater dispersion compared to the vertical rectus and superior oblique. There was no statistically significant difference between extraocular muscles (p-value > 0.05).
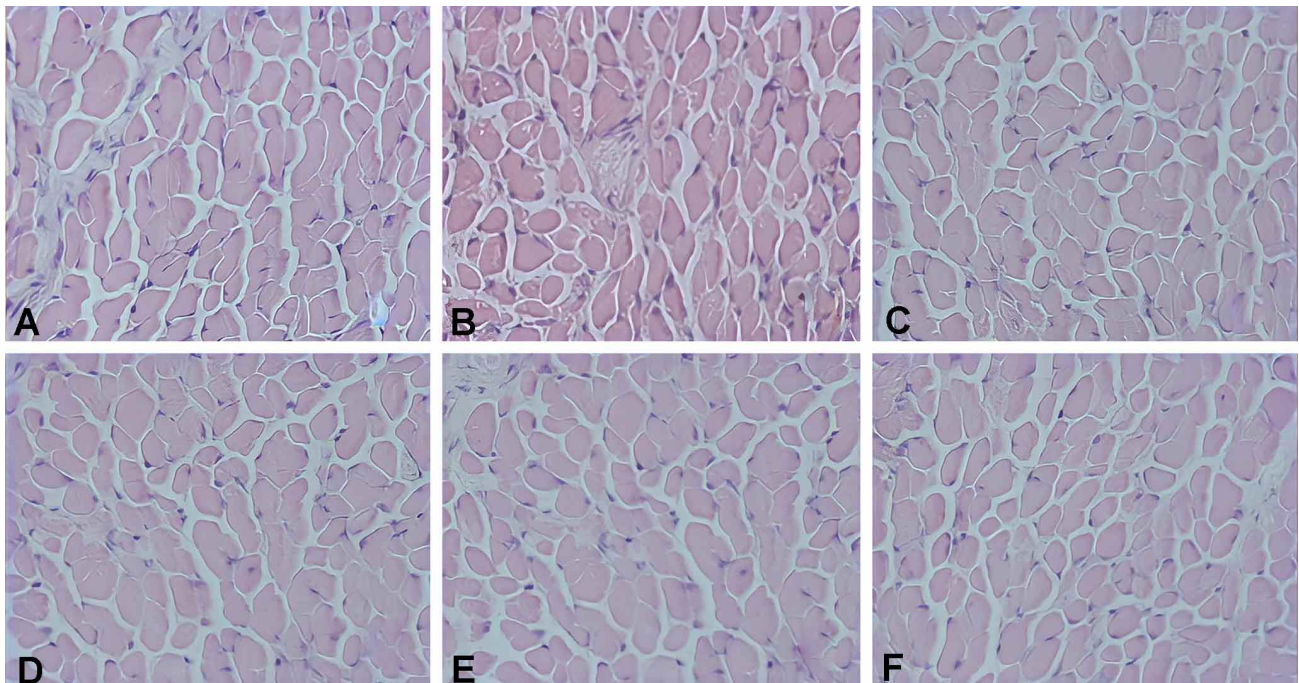

Fig. 4. Optical microscopy, Hematoxylin-Eosin (40X): Each plaque corresponding to a different extraocular muscle: inferior oblique (a), superior oblique (b), inferior rectus (c), lateral rectus (d), medial rectus (e) and superior rectus (f).

DISCUSSION

The stereological method allows the study of three-dimensional biological structures by analyzing two-dimensional histological sections, giving as result reliable numeric parameters. This technique is being used more in a wide range of biomedical fields and is considered the gold standard in quantitative morphometry (Howard & Reed, 2005; Mühlfeld *et al.*, 2010; Mühlfeld & Ochs, 2013; Mandarim-de-Lacerda & del Sol, 2017). The present study describes the normal histology of the extraocular muscles using stereology and specifically determines the proportion of muscle and collagen tissue and quantifies the number of muscle fibers in 5,000 μm^2. To our best knowledge, there are no previously published
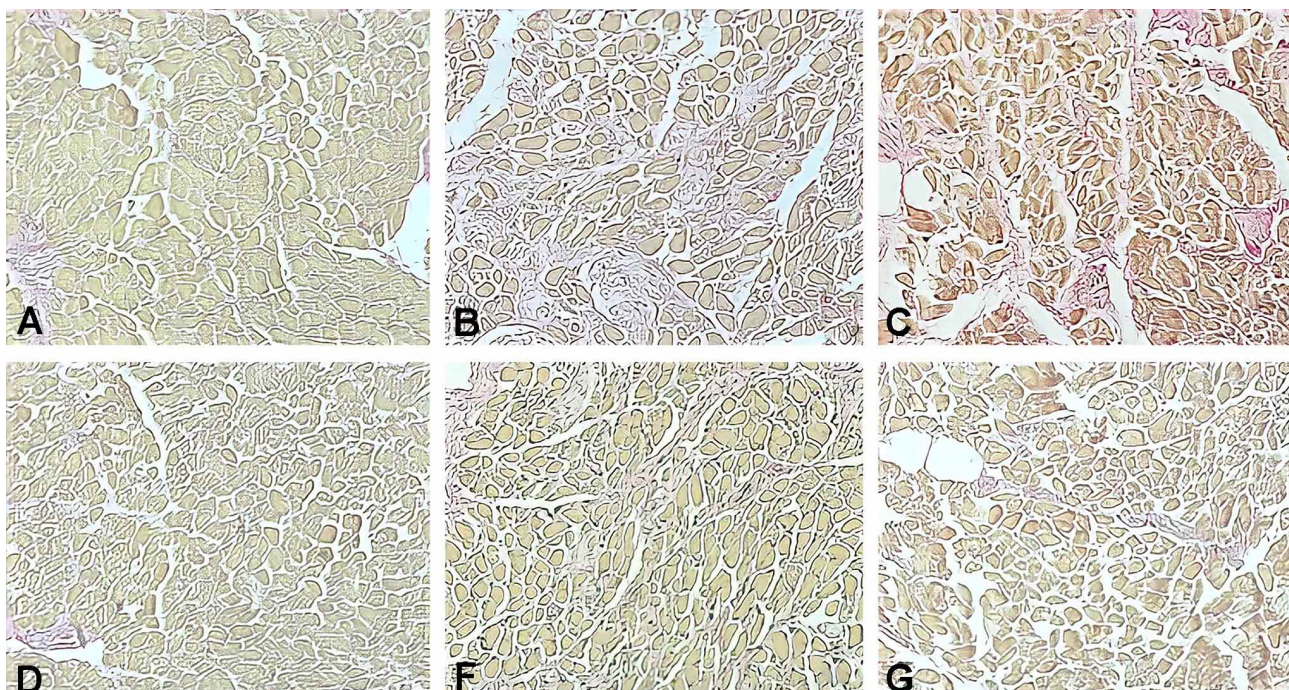
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Fig. 5. Optical microscopy, Van Gieson (40X): Each plaque corresponding to a different extraocular muscle: inferior oblique (a), superior oblique (b), inferior rectus (c), lateral rectus (d), medial rectus (e) and superior rectus (f).

studies in which these variables are described in humans, using stereology.

Regarding our results, it is interesting to mention how the histological parameters we studied have a morphological – functional correlation. The horizontal rectus (medial and lateral) showed higher proportion of muscular volume density (Vv) when compared to cyclovertical muscles (vertical rectus and oblique muscles). This is somewhat predictable because lateral versions are the most frequently executed eye movements. The medial rectus showed the highest muscle Vv, a result that is also expected because it is involved in both convergence and lateral movements. Nonetheless, those differences did not achieve statistical significance. Certain level of similarity is expected between the extraocular muscles considering that the oculomotor system is highly balanced, in which yoke (synergistic) and antagonist muscles have been clinically described (Hering *et al.*, 1977; Davis *et al.*; Bruenech & Haugen, 2015).

Unfortunately, there are no published stereological studies regarding extraocular muscles histology, in both animals and humans. Several studies have employed stereology for the description of limbs muscle fibers in humans, but they all analyze ultrastructural features, such as mitochondrial volume density (Broskey *et al.*, 2013; Picard *et al.*, 2013). Thus, there is no available data for our results to be compared.

The present study has certain limitations among which are the limited number of individuals analyzed, representing only a specific age range and ethnicity. Additionally, we only described three histological parameters and we do not distinguish between muscle fiber types and collagen subtypes.

CONCLUSION

This study establishes normal stereological parameters in extraocular muscles of humans without ophthalmological disease. This data will allow future comparisons if similar studies are performed. It would be particularly interesting to see if there is any difference in these stereological parameters in the extraocular muscles of strabismic humans.

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RESUMEN: El objetivo de este estudio es cuantificar el volumen de tejido muscular y conectivo de los músculos extraoculares en humanos sin enfermedad oftalmológica conocida utilizando estereología. Los músculos extraoculares fueron obtenidos de cinco cadáveres humanos sin estrabismo. El número de fibras musculares en 5.000 µm2 y la densidad de volumen (Vv) de

músculo y colágeno fueron medidas usando estereología. Las comparaciones entre músculos extraoculares antagonistas se realizaron a través de la prueba de los rangos con signo de Wilcoxon para muestras pareadas. Un análisis secundario examinando diferencias entre pares de músculos extraoculares también fue llevado a cabo. Se realizaron pruebas bilaterales y la significancia fue fijada en 0,05. Los músculos rectos horizontales (recto medial y lateral) tuvieron el mayor Vv de músculo y el menor Vv de colágeno. El músculo recto inferior tuvo la tendencia a poseer menos número de fibras por 5.000 µm^2 que el resto de los músculos extraoculares. No obstante, estas diferencias no llegaron a ser estadísticamente significativas. Este es el primer estudio publicado describiendo la histología normal de los músculos extraoculares usando estereología. Nuestra investigación, a través de la cuantificación de la proporción de tejido de músculo y colágeno, así como el número de fibras musculares en 5.000 µm^2, establece parámetros estereológicos normales para músculos extraoculares en humanos sin enfermedad oftalmológica.

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