Geographical dimensions of fibers from the soleum muscle in rats exercised on treadmill: the importance of the analysis by means of digitalized images*

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ABSTRACT

The purpose of this paper was to assess a new methodology to analyze digitalized cross sectional images from the skeletal muscular fibers in rats submitted to physical exercise on treadmill. It was used portions of the soleum muscle of rats attained from histological cuts and tinted with hematoxylin and eosin (HE). 100 muscular fibers were assessed from each animal, and their perimeter, the area, and the maximal, medium and minimal diameters were measured by means of the segmentation process from digitalized images of the fibers' sections using the software Image-Pro Plus. The geometrical dimensions such as the area, the perimeter and the medium and minimal diameters of the cross section of muscular fibers revealed to be adequate to analyze the effect of the training in rats. The analysis revealed the existence of an interaction between the group of rats and the duration of the physical exercise. The Pearson's correlation coefficient was higher between the medium diameter and the area of the fibers (0.97), followed by the correlation between the maximal and medium diameter with the perimeter (0.93). It can be concluded that the measurement of the grade of the hypertrophy of the muscular fibers can be performed by determining the medium diameter or the cross sectional area of the fiber, and thus constituting an adequate and effective methodology especially for muscular fibers with accentuated polymorphism.

Keywords: Muscular fibers. Physical training with animals. Video microscopy.

INTRODUCTION

The functional overload induced by physical exercising promotes an increase in the muscular tension and power production, making the skeletal muscle susceptible to alterations[1,2]. Along the more advanced phases of the exercise, a noticeable adaptation is the hypertrophy of the muscular fibers[3,4], for instance, like those that happen in the quadriceps muscle in individuals submitted to the resistance and strength training[5].

One way to study the alterations in the muscular fibers, such as the hypertrophy, it is measuring the geometrical dimensions[6] of the cross section of the fibers. A similar technique has also been used to study the detection of cancerigenic cells[7] and the morpho-functional characteristics of the fibers in the skeletal muscle in species of animals with economical interest, with the purpose to attain information on the quality of their meal[8-10].

In the morphometric studies of the muscular tissue the geometrical dimensions of the cross sectional fibers, like the orthogonal diameters, the perimeter of the section as well as the area are frequently assessed[11,12]. One of the methods used to make a quantitative analysis of the fiber is to measure its lower diameter, as it was proposed by Dubowitz[13]. That methodology has been widely used in assessing the induced hypertrophy by physical exercising and in the atrophic processes induced by the lack of use or denervation[12]. The study of the cross sectional geometry of the muscular fibers, as it was proposed by Dubowitz, has been performed through the ocular reticulum, and using drawings in clear chambers coupled to an optical microscope. This methodology is a set of accentuated time-consuming slow procedures that leads to sometimes dependent results in part due to the researcher's subjectivity. The usage of digital images is demanding in researches in areas such as biology, for medical diagnosis, in the remote monitoring, in astronomy and automation, etc.[14].

The present paper has as main purpose to assess the application of the morphometric method through the use of computerized analysis of digitalized images, to study morphological alterations in cross sectional fibers of the soleum muscle in rats submitted to physical exercising on treadmill.

This methodology has as major aim to attain an objective process as to simplify and mathematically quantify the measurement of geometrical dimensions in the cross section of muscular fibers.

MATERIALS AND METHODS

The images used in this study were attained using plates from muscular fibers prepared in a previous work made by one of the authors[15]. As the procedures to attain the images are important for the work assessment, a resume of the training in rats was performed, as well as the preparation of the images from the muscular fibers. 30 Wistar rats (Rattus novergicus, albine variety) aged from 100 to 180 days were used, and they were kept in plastic 30 x 16 x 19 cm cages, having five rats per cage at 22°C mean temperature, 12 hours claire-obscure cycle, the claire cycle starting around 7 a.m., and they were fed with a standard ration and free water. The rats were randomly divided in six groups (three groups were denominated *exercised group* and three group of rats as the *control groups*), five rats per group, and therefore characterized as a factorial and completely random 2 x 3 trial. The rats in the exercised group were submitted to physical training on treadmill in two steps: the first step was called adaptation phase, when the rats were submitted to daily sessions of exercises on the treadmill, lasting 5, 15, 30, 45, and 60 minutes during the 5 first days of the trial, and the training phase with daily 60 minutes duration exercising sessions five days per week between 2 p.m. and 5 p.m. The three groups of exercised rats were sacrificed after 30, 45, and 60 training days. The control rats were submitted to the

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same procedures than the exercised rats, except that they were not submitted to the physical exercise on treadmill.

To the data collection, the rats were sacrificed with an injection containing 20 mg/100 mg of the body weight sodic pentobarbital[16] and the surgical procedure started immediately to withdraw the soleum muscle of the right pelvic member. Approximately 2 cm length and 0.5 cm diameter samplings of the soleum muscle from the abdomen with longitudinal fibers disposed along the high length axle were frozen in N-Hexana, and they were cooled at –70°C[17]. Next, 8 µ width histological cuts were made in a cryostat microtomy model HM 505 E Microm, perpendicular to the major axle of the fibers at –20°C temperature, and next, they were tinted using the hematoxylin and eosin method (HE)[18].

The images of 100 fibers (cross sectional) of each rat were digitalized with a 50X augmentation using a digital camera coupled to an optical Leica microscope, and their dimensions were gauged using a micrometric rule. According to the justification presented in the session "Discussion", it was used only 100 fibers that represents the half amount that has been often used in the literature.

To assess the dimensions of the muscular fibers, it was used the Image-Pro Plus version 4.5 software that allows to segment the cross sections of the muscular fibers' images, thus attaining their geometrical dimensions. The software supplies 53 parameters of each image, such as color, intensity, density of the image, and geometrical dimensions. In this study, the parameters related to the color, density and intensity of the images are irrelevant. The determination of the minimal, maximal and medium values of the diameters is mathematically performed as follows: initially, the software sets the positioning of the centroid of the picture corresponding to the cross section of the fiber.

After that, it is determined the length of the set of straight lines (diameters) that pass through the fiber's centroid, uniting two points of the fiber's perimetral curve. From the set of straight lines, the software determines the values related to the length of the minimal, maximal and medium diameter. Other mathematical variables of the fibers' cross section supplied by the software are: form factor, the angles of the main axles, and the fractality of images. In this study, these variables were not considered, since they did not represent significant variations in the trial using rats.

To analyze the fibers, it was selected the following geometrical dimensions: area, perimeter, and minimal, maximal, and medium diameters, whose values were stored on an Excel worksheet. The statistical analysis of the results attained as geometrical dimensions was performed through the software Statistical Analysis System – SAS. In order to study the comparison between groups of rats and exercising periods, it was applied the Two-Way ANOVA variance analysis, and thus attaining the effect in the groups of rats, the training days, and the interaction between days and groups. For the dimensions presenting significant variations, it was applied the Tukey's test of multiple comparisons with 5% significance level. This procedure was adequate when it is considered the utilization of a factorial and completely randomized trial. The results follow a normal distribution (Shapiro-Wilks test with 5% significance level), and the groups are independent[19,20]. The homogeneity of the variances was not tested, as the variance analysis is considered a strong technique[21].

RESULTS

Figure 1 shows a cross section of the soleum muscle from an animal submitted to 30 days of physical training. It can be observed that the muscular tissue has a delimited normal fascicular pattern through the perimysium, and each fiber is surrounded by the endomysium. The fibers have polygonal outlines, with one or more nucleus in peripheral positions. It was not shown images of fibers from the control rats, since they did not present any significant visual difference compared to images of the fibers seen on figure 1.

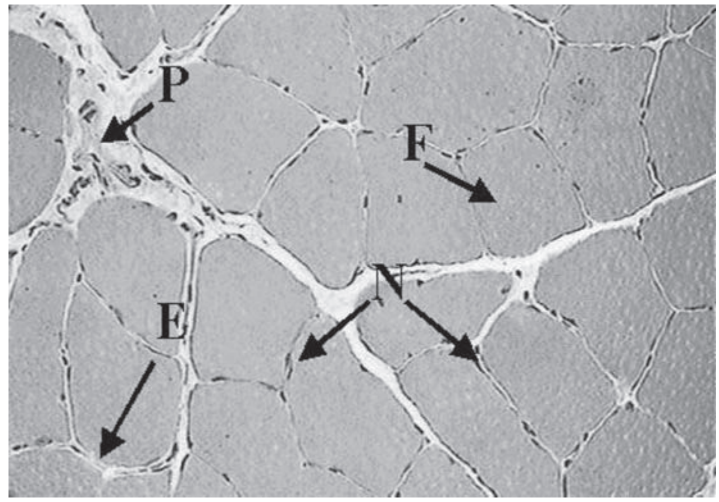

Figure 1 *– Cross section of the soleum muscle of exercised rat (30 days). It can be observed on the figure that the sections of the fibers are polygonal (F) and have peripheral nucleus (N), perimysio with blood vases (P), and endomysio (E). HE. 50X.*

Figure 2 shows an example of digitalized image of a cross section of the soleum muscle showing images of fibers in a dark tonality being worked on the environment of the Image-Pro Plus software. The numbers shown on the picture identify each of the already segmented fibers, whose dimensions are stored on the software's worksheet, and which were later exported by the software in the Excel format.

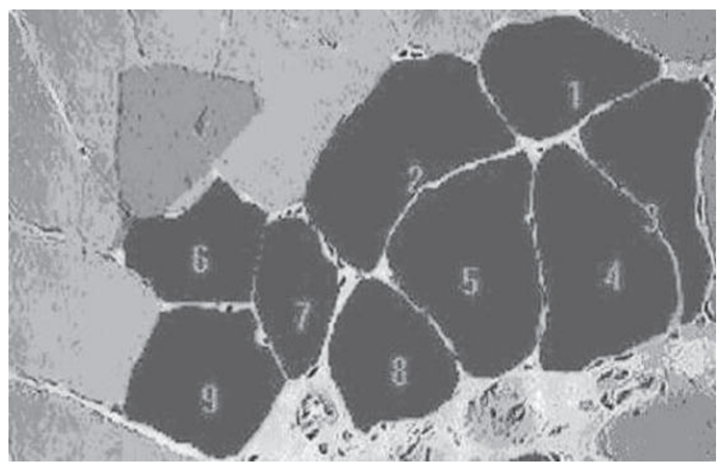

Figure 2 *– Cross section of the soleum muscle of exercised rat (60 days). In dark tonality, it is shown the delimited fibers using the Image-Pro Plus software. The numbers 1 to 9 identify the delimited fibers (1 to 9). HE. 50X.*

As example of the results, table 1 shows the results attained as to the following dimensions: maximal, minimal, and medium diameter of the cross sections of the muscular fibers of rats from the trained groups at different times, and the corresponding results attained for the control groups. According to the data presented on table 1, those groups submitted to the physical training presented systematically higher values of the diameters than those observed in the control groups.

The variance analysis was applied to compare the means between groups of exercised and control rats, and it was attained the p-value < 0.05 in every comparison performed, that is, the differences between them was significant at 5% level. Still, the variance analysis has shown that there is an interaction between groups of rats and the duration of the exercise, that is, their effects along the time were different for both assessed groups.

TABLE 1
Mean and standard deviations of the dimensions of the medium diameter (Dmed.), minimal diameter (Dmin.), and maximal diameter (Dmax.) of the cross sections of fibers from trained and control rats

		30 Days µm	45 Days µm	60 Days µm
Dmed.	Trained	68 ± 11[a]	68 ± 13[a]	69 ± 13[a]
	Control	63 ± 10[b]	66 ± 12[c]	60 ± 11
Dmin.	Trained	48 ± 10[a,d,e]	50 ± 10[a]	50 ± 10[a]
	Control	46 ± 9[b,c]	49 ± 10	43 ± 9
Dmax.	Trained	94 ± 21[a,d]	91 ± 21[a]	92 ± 22[a]
	Control	84 ± 19[b]	88 ± 20[c]	80 ± 18

[a] Statistically different means related to the respective control group; [b] Same related to the 45 days control group; [c] Same related to the 60 days control group; [d] Same related to the 45 days exercised group; [e] Same related to the 60 days exercised group.

For exercised rats, it was found significant differences as to the maximal diameter between 30 and 45 days, while the minimal diameter, added to the difference observed between the 30th and the 45th day it appeared also a difference between animals in the 30th and the 60th day of life. As to the medium diameter, it was observed no significant difference between the training days. Among the control rats, there were significant differences of means in the medium and maximal diameters between the 30th and 45th day, and for the minimal diameter, there also have been differences between the 30th and the 60th day.

Table 2 contains the results of the statistical analysis for the following dimensions: area and perimeter of the cross sections of the muscular fibers in trained rats in different training times and the corresponding results on the fibers in control rats. It can be observed a statically significant increase in the area and perimeter in rats submitted to the physical training compared to their respective controls in every considered time. The variance analysis showed an interaction between groups of rats and the duration of the exercising, that is, the response profile between groups of rats, when it is considered the training days varied along the time between groups of rats.

TABLE 2
Means and standard deviations of the dimensions of the area and perimeter of the cross section of the fibers of trained and control rats

		30 Days µm²	45 Days µm	60 Days µm
Area	Trained	3,568 ± 1,157[a]	3,640 ± 1,242[a]	3,661 ± 1,312[a]
	Control	3,108 ± 1,044	3,372 ± 1,119	2,797 ± 1,027
Perimeter	Trained	247 ± 43[a]	243 ± 48[a]	247 ± 47[a]
	Control	226 ± 43[b]	236 ± 43[c]	216 ± 41

[a] Statistically different means related to the respective control group; [b] Same related to the 45 days control group; [c] Same related to the 60 days control group.

Trained rats did not present significant alterations in the area and perimeter along the training period, while in the control rats the means of the perimeter has presented significant differences at the 5% level between the 30th and 45th day, and between the 45th and 60th day, and the area did not show significant differences.

Table 3 shows the Pearson's correlation coefficients between geometrical dimensions of the cross sectional of fibers. However, through the Pearson's correlation test, every coefficient calculated is significant (p-value < 0.01). Nevertheless, it is observed a high value for the Pearson's coefficient between the medium diameter with the area, and of the medium diameter with the perimeter. Furthermore, it was observed that the correlation coefficient between dimensions of the maximal and minimal diameter was lower. This fact may be directly related to the cutting plan used in this trial. In a general way, these results show a positive association between the considered measurements. One must be careful when making such interpretation, since a positive association does not necessarily mean that every measurement is coincident, but indicating only that they are correlated.

TABLE 3
Pearson's correlation coefficient between geometrical dimensions of the fibers

	Dmax.	Dmin.	Dmedio	Area	Perimeter
Dmax.	1.00	0.33	0.87	0.81	0.93
Dmin.		1.00	0.68	0.76	0.54
Dmed.			1.00	0.97	0.93
Area				1.00	0.89
Perimeter					1.00

Note: To check if the correlation is null in the hypothesis test, we attained p-value < 0.01.

DISCUSSION

The analysis of this work shows five dimensions of the geometrical form of the fibers' cross section of the soleum muscle: the maximal, minimum and medium diameter, as well as the area and perimeter increased in rats submitted to physical training compared to their respective control rats, indicating that there was a muscular hypertrophy. Modifications in the size and amount of the muscular fibers in rats submitted to physical exercises were also reported by Paul and Rosenthal[22] and Giddings *et al.*[23].

The measurement of the fibers' size, especially in subtypes, has been proved to be essential to the diagnosis[7,13] to analyze the muscular biopsies associated to different pathologies and in experimental conditions, such as the hypertrophy caused by repetitive stress and sustained work, atrophy due to the lack of usage, by denervation and longitudinal division (splitting) of the fibers. The hypertrophic process in the muscular fiber can appear within an interval of months, but the skeletal striate muscle can attain the hypertrophy state in short intervals of time, as it was observed in this work[22-24]. The alterations observed in the geometrical dimensions of the cross section in the control rats along the time can be partially attributed to the aging of rats.

In order to study the morphometry of the muscular fibers' cross section in rats, the methodology used in this paper shows two advantages related to the visual analysis process of the fibers' analysis: the first one is the agility of the process, since it was attained a reliable static result using a lower total amount of fibers, as it was proposed by Dubowitz[7,8], and the second one is the elimination of the researcher's subjectivity.

The geometrical dimensions are determined through the mathematical analysis of the fibers' outline, differently from the Dubowitz's work, who used a more subjective measurement[15,25,26].

From the analyzed geometrical analysis, it is observed a close to 1Pearson's correlation coefficient between the medium diameter and the area, and a lower figure for the maximal and medium diameters with the perimeter, indicating that the medium diameter and the area are the most indicated dimensions to measure muscular fibers. The utilization of the medium diameter of fibers as an analysis parameter aims to overcome mainly the variability of the muscular fibers with an accentuated polymorphism, where the measurement of the lower diameter lead to less consistent and hard to be reproduced results[15].

According to the Dubowitz's criterion[13,25,26], it is often used around 200 muscular fibers, from which is measured the lower diameter at a central spot or close to the core of the fiber, in an orthogonal positioning related to the higher axle of the fiber. In this paper, the analysis performed has also aimed the minimal amount of fibers that must be analyzed, in order to achieve reliable results. Setting a 95% confidence level and 0% maximal relative error, the conclusion set that a 100 fiber sampling per animal is a sufficient amount for this type of study. Due to this, our analysis was made with that amount of fibers.

The mathematical value of the geometrical dimensions of the fibers' sections in this research can have a different meaning from the lower diameter proposed by Dubowitz(13). Nevertheless, the five determined variables show that they could be used to assess the hypertrophy level of the muscular fibers after physical exercises.

There was a major correlation factor between the values of the medium diameter and the perimeter, and they can be safely chosen to the morphometry of the fibers. It is intuitive that if the cut of the fibers is cross sectional to the major axle of the fibers, it could be used in the morphometric study. Such fact is mathematically expected, since all these geometrical dimensions are proportional to each other. It remains as suggestion for future papers testing this methodology in histological cuts intentionally performed as not be perpendicular to the length of the muscular fibers.

CONCLUSION

The application of the present mathematical methodology allowed to safely, effectively, and quickly assess the hypertrophy of fibers in rats exercised on treadmill. Although the measurement of the lower diameter of the fibers described by Dunowitz(13) represents a safe criterion in this investigation field, our results show that other geometrical dimensions of the fibers, such as the medium diameter, can also be used. For this, it is necessary that the orientation of the cutting plan of the tissue during the microtomia is perpendicularly made to the axle along the length of the fiber.

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