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SHORT-TERM IMMOBILIZATION CAUSES MORPHOMETRIC AND MECHANICAL ALTERATIONS ON RAT MUSCLES

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ABSTRACT

Objective: to analyze the morphometric and mechanical characteristics of the soleus and gastrocnemius muscles after immobilization in a shortened position. Methods: 20 Wistar rats (250 ± 20g) were divided equally into immobilized and control groups. The left hind limb was immobilized by means of an acrylic resin orthosis, with the ankle joint at maximum plantar flexion. After seven days of immobilization, the muscle mass, number and length of sarcomeres in series, muscle fiber cross-sectional area, density of the intramuscular connective tissue area and tensile strength of the triceps surae muscle were evaluated. The data were analyzed by the ANOVA and Tukey tests ($p < 0.05$). Results: The immobilized soleus muscle presented changes in all the morphometric variables analyzed, while some of these changes were not observed in the gastrocnemius muscle. Analysis of the mechanical test showed that the immobilized group presented a 20% decrease in the maximum tensile muscle strength. Conclusion: The results from this study showed that short-term immobilization causes changes to the morphometric parameters of the muscle fibers, with repercussions on muscle mechanics. These results suggest the need for rehabilitation of muscles subjected to immobilization, even if only for a short period, in order to achieve early recovery of normal muscle characteristics.

Key words: immobilization; skeletal muscle; mechanical test; morphometry.

INTRODUCTION

Muscular tissue has a notable capacity for structural and functional adaptations after of several stimuli. One of these stimuli is immobilization, a procedure commonly used as a form of treatment of muscular-skeletal injuries. There are many effects of immobilization on skeletal muscle, and the most noticeable are the reductions in muscular glycogen reserves, intramuscular connective tissue proliferation, muscular atrophy, alterations of the number of sarcomeres in series and decreases in muscular strength[1-5]. Furthermore, modifications of the mechanical properties of the skeletal muscular system submitted to movement restriction have been reported[6,7].

The muscular adaptations in response to immobilization are influenced by the position in which the muscle is immobilized. In a certain way, the shortened position is the one that yields greater tissue adaptations, when compared to the neutral or stretched positions[8,9]. The adaptations that occur after immobilization are influenced not only by the joint position, but also depend on the muscle involved, the oxidative ones being more affected than the glycholitic[9,10].

Previous works have shown connective tissue proliferation increases, fiber length reduction, muscular atrophy and decreases in muscular mechanical properties after two or three weeks of shortened position immobilization[4,7,11]. On the other hand, there are studies which show that some of these adaptations can already be observed between one and seven days[6,12].

The aim of the present study was to assess if only one week of immobilization was enough to lead to adaptations to the sarcomeres and morphometry of the soleus and gastrocnemius fibers, as well as alterations in the mechanical properties of the sural triceps muscle.

METHODOLOGY

Subjects

Twenty-one Wistar rats were used, with a corporal weight of 250 ± 20 g. The animals were kept in a biotherium under controlled conditions, with a light/dark cycle of 12 hours, temperature 23º ± 2º, with free access to water and food. The project was approved by the Animal Ethics Committee of the Federal University of São Carlos (protocol nº 15/06).

Animals were randomly distributed in two groups: immobilized (n= 10) and control (n= 10); five animals of each group were used for mechanical test, and five for soleus and gastrocnemius muscle analysis. For the joint immobilization procedures and muscle removing, animals were anesthetized with intramuscular injections of ketamine chloridrate (50 mg/mL) and thiazine chloridrate (2 g/100mL), in the proportion of 1:1, at a dose of 0.1 mL/100g of corporal weight. All animals were sacrificed by means of cervical displacement.

Procedure for joint immobilization

After anesthesia, animals were submitted to immobilization of the left posterior limb by using an acrylic resin orthosis model proposed by Silva et al[13]. Animals were immobilized during seven days, with the soleus and gastrocnemius muscles kept in the shortened position, with maximal ankle flexion, at approximately 170º.

Procedure for traction mechanical test

After the animals were sacrificed, the left posterior limb of the same were dissected and disarticulated from the femoral thigh. The mechanical features of the sural triceps muscle were analyzed by means of mechanical test of traction on the universal test machine DL 2000 (EMIC – Brazil). In order to do so, the tibial and fibular muscles were removed, and the soleus and gastrocnemius muscles were positioned longitudinally over a device confectioned for the femoral and calcaneus (Figure 1). The tests were standardized with a initial pre-tension of 0.15 N, the traction had a velocity of 5 mm per minute, with the measurements performed every 0.5 mm.

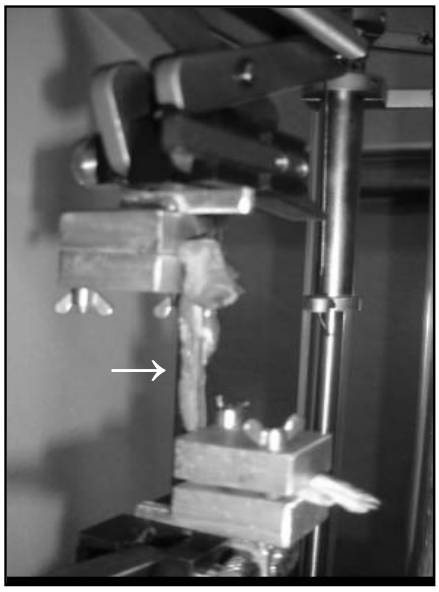

Figure 1. Lateral view of mechanical test, identifying the site of the muscle rupture (arrow).

Morphometric analysis

Animals previously anesthetized had their soleus and gastrocnemius muscles removed, weighed and divided longitudinally into two parts, one for a morphometric evaluation (connective tissue area density analysis, and the fiber cross sectional area) and the other for the sarcomere adaptation analysis (number and length of the sarcomeres). For the morphometric analysis, the midbelly portion of the muscle was fixed with pins over cork plates, and was frozen by immersion in liquid nitrogen-cooled isopentane. Muscle cross sections (12 µm thick) were obtained in cryostat microtome 300 (ANCAP – Brazil) and stained with Hematoxiline and Eosine (H&E). The best histological cut, free from artifacts and blood vessels, was chosen and photographed with a 20x objective lens in full extension. Images were obtained by means of a BX-41 optical microscope (Olympus – Japan) attached to a C5050 digital camera (Olympus – Japan), with the connective tissue area density measured by point-counting planimetry[14], using the Image Pro Plus 4.0 software (Media Cybernetics – USA). The cross section area (CSA) of approximately 200 fibers/ muscle was measured using the software Motic Image Advanced 3.2 (Motic Instruments – Canada).

For the sarcomere adaptations analysis, muscles were attached to cork plates with pins in the rest position, and fixed in 2.5% gluteraldehyde for three hours. After this period, samples were placed in 30% nitric acid for three days, and then kept in 50% glycerol until the moment of analysis[15]. All muscles had their lengths obtained using a calipers. In order to measure sarcomere number, five fibers of each muscle were removed and six fields/fibers were photographed. From each field, the number of sarcomeres was counted throughout 50 µm, summing a distance of 300 µm/fiber. All images were captured with a 100X lens. The total number of sarcomeres in each muscular fiber was determined through correlations between the number of identified sarcomeres at a distance of 300 µm and total muscle length[16], and the average length of each sarcomere was obtained by dividing the muscle length by the number of sarcomeres.

Statistical analyses

Initially statistical analyses were performed by the Kolmogorov-Smirnov normality test and by the homocedasticity test (Bartlett's criterion). After the observation that the variables were compatible with parametrical analyses, ANOVA, and F tests were used. When the differences were significant, Tukey's HSD test was applied for multiple comparisons. For all calculations, a significance level of 5% was established.

RESULTS

The immobilized soleus muscle showed reduction of 35% of its muscle mass, and 21% of the number of sarcomeres in series, when compared to the control ($p < 0.05$; Table 1). The immobilized gastrocnemius showed reduction only in the number of sarcomeres, and this was 10% in relation to the control muscle ($p< 0.05$; Table 1). In relation to the sarcomere length, there was observed a 5 to 6% increase for the immobilized gastrocnemius and soleus muscles, respectively, when compared to their respective controls ($p< 0.05$; Table 1). For the muscle length, only the soleus showed a 16% reduction in relation to the control ($p< 0.05$; Table 1). The immobilized soleus and gastrocnemius muscles showed TAS decreases of about 13%, connective tissue area density increases of 50% and 132%, respectively, when compared to the control muscles ($p< 0.05$; Table 1 and Figure 2).

Mechanical test analyses did not allow the assessment of the behavior of the soleus and gastrocnemius muscles separately, but from the sural triceps as a whole. The results showed a 20% reduction for maximum rupture strength on the immobilized group, when compared to the control ($p< 0.05$, Table 1). The site of the rupture occurrence of all muscles was on the midbelly of the muscle portion.

Table 1. Average ± reliability intervals of the variables from all experimental groups.

Variables	CS	CG	IS	IG
Muscle mass (mg)	120 ± 4.5	641 ± 69.9	78 ± 5.5*	599 ± 48.3
Sarcomere number	7181 ± 679	12132 ± 514	5684 ± 772*	10860 ± 495*
Sarcomere length(μm)	2.02 ± 0.1	2.14 ± 0.08	2.14 ± 0.17*	2.24 ± 0.1*
Muscle length (mm)	14.2 ± 0.7	25.8 ± 1.1	12 ± 1.06 *	24.2 ± 0.96
CT area density (%)	15.64 ± 1.4	9.52 ± 2.2	23.53 ± 6.06*	22.07 ± 5.5*
Muscle fiber TAS (μm^2)	2637 ± 601.2	2800 ± 252	2302 ± 688*	2414 ± 177*
Rupture maximal strength (Kgf)	47.02 ± 2.6		37.74 ± 3.0*	

*when compared to the control (p< 0.05). Connective tissue (CT); cross section area (CSA); control soleus (CS); immobilized soleus (IS); control gastrocnemius (CG) and immobilized gastrocnemius (IG).

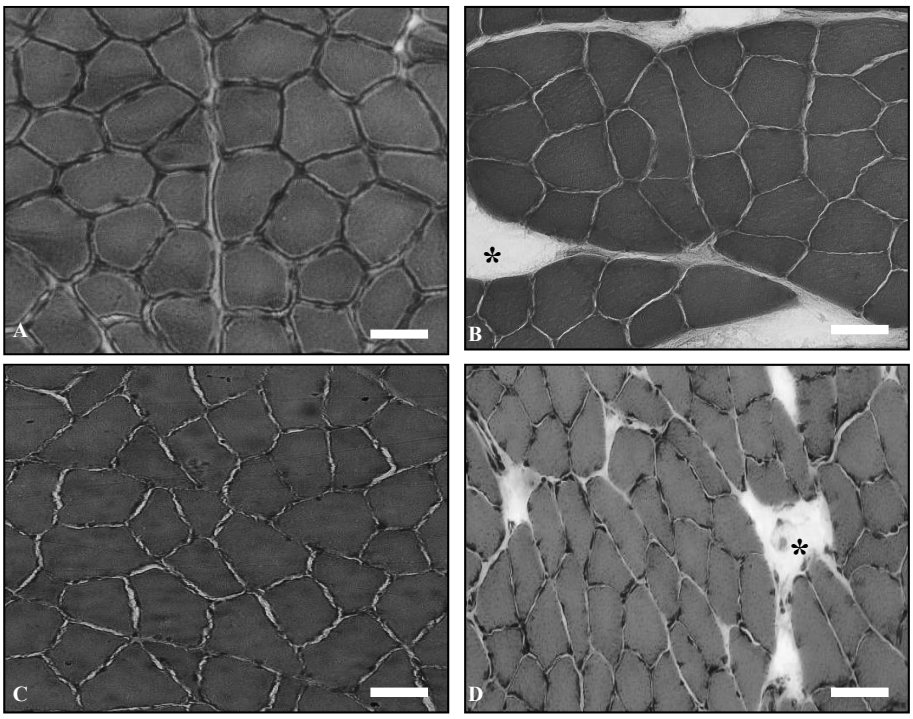

Figure 2. Cross section of the gastrocnemius and soleus muscles of control (A, C) and immobilized group (B, D). Note the increases in the perimysial connective tissue density in the immobilized muscles (*) H&E, 20x. Bar = 50μm.

DISCUSSION

The results from this study show that the short period of immobilization, of only one week, was enough to promote important sarcomere adaptations, and morphometric and mechanical alterations of the soleus and gastrocnemius muscles of rats. However, it is important to consider that, although the analyzed variables showed coherence between them, the present results should be considered cautiously, due to the small samples (five animals/group).

The understanding of the elastic properties of a tissue is of great importance to rehabilitation professionals, because this knowledge helps diagnosis, treatment and prevention of sport and orthopedic injuries. The mechanical test is very often used as a way of studying these properties, since it is easily used, and allows reproducible results.

Among the important biological tissues studied by mechanical test, are the tendons[17], ligaments[17,18] and muscles[6,7,19]. Differently from the tendon, which is capable of tolerating high tensions without the loss of fiber integrity, the skeletal muscle, when submitted to high tensions, responds with fiber breaking. The mechanical test studies of muscular traction focus, basically, on the influence of stimuli such as immobilization on the mechanical properties of the muscles. It is known that immobilization alters the elastic properties of muscles, which causes muscle fibers ruptures with lower strength[6,7,19].

According to Jarvinen[19], the rupture points of the gastrocnemius muscle, when submitted to mechanical traction test, varied very little, since in 94% of the muscles the ruptures occurred on the midbelly portion. The results found in the present study are in accordance with these findings, once the muscular rupture site of all assessed muscles was located at the midbelly of the muscle. One of the hypotheses for injury susceptibility at the midbelly region is that it has higher muscular tissue concentrations than the connective tissue which is considered the most fragile site of the muscle.

It is important to highlight that, although the results of the present study show higher connective tissue density areas in the immobilized muscles, this increase did not cause higher muscular resistance to the mechanical test. This happened because the area density of the connective tissue does not seems to be a determining factor to resist increases in the tissue stress, since the organization of this tissue is the feature that contributes the most for the muscle's capacity to resist stress. According to Lieber et al.[20], the muscles of individuals with spasticity demonstrate high extracellular matrix densities, however, with inferior mechanical capacity due to the limited organization of its extracellular matrix.

Thus, although in the present study a qualitative analysis of the conjunctive tissue has not been made, the results allow suggestions that the animals that were immobilized for a week showed poor organization of this tissue, because they supported less strength needed for muscular rupture. Proliferation of intramuscular connective tissues after immobilization seems to be related to the decrease of muscular contractile activity[16], since the increase of area density of this tissue rapidly occurs, only after two days of immobilization, preceding the adaptations that occur to the sarcomeres[21]. Not only does the contractile activity decrease in the immobilized muscle, but there is also a mechanical load decrease. This also affects the proliferation of this tissue, once it regulates the production of growth factors and collagen synthesis[22]. In this sense, fibroblasts, which are subject to active and passive muscular tension, have their metabolism altered with the decreases of tension caused by immobilization, which incurs in area density increases of the intramuscular connective tissue.

The sarcomere length can also be a variable that contributes to decreases of muscular rupture maximal strength, once the increase of length of the same makes the sarcomeres become tenser, therefore more susceptible to injury[10]. In the present study, there was an increase of sarcomere length in both immobilized muscles, which shows short-term sarcomere adaptation after immobilization.

Concomitantly with sarcomere length increases, there was a decrease in the number of the same. This adjustment occurs so that there is an ideal actin and myosin filament overplacement, which allows optimal tension development during contraction[23]. This adaptation of the sarcomere numbers is needed, once the myosin histochemical and actin filaments possess constant length. Thus, the way to cause optimal overplacement, after a period of immobilization, is altering the number of sarcomeres.

While both immobilized muscles, soleus and gastrocnemius, have shown CSA decrease in their fibers, only the soleus muscle showed muscle mass reduction. The larger compromising of the soleus after immobilization may be explained by at least two hypotheses: the difference in the types of fibers, and the difference of anatomical position between the muscles. It is known that type 1 fibers, with predominant oxidative metabolism, are the most vulnerable to disuse-induced atrophy[2,10], and the soleus muscle shows higher proportions of these fibers than the gastrocnemius muscle. Not only there is a histochemical difference between fibers of these muscles, also the gastrocnemius (bi-articular muscle) has suffered less effects from immobilization when compared to the soleus muscle (mono-articular), since in the immobilization model used, the knee articulation of the animal remains free.

Although it is well established that muscular atrophy is characterized by decreases of protein content, fiber diameter, muscular strength production, and fatigue, the cell and molecular mechanisms involved in the muscular disuse-atrophy installation are not completely determined[24,25]. However, it is known that the disuse-atrophy process starts with the reduction of muscular tension, which will reflect in both protein synthesis and degradation, and the mechanisms

related to proteolysis are more known than the ones involved in proteic synthesis decreases[26]. The proteolythic systems involved in muscular atrophy are: the calcium-dependent calpain system, the lisossomal proteases (cathepsin), and the ubiquitin-proteossome system[24,26]. The initial action of the calpain system is necessary because the ubiquitin-proteossome system does not degrade intact myofibrils. In this case, a prior protein disarrangement is necessary for posterior proteolysis. Because calpain has the titin protein as a substrate, the initial activation of this system yields sarcomere misalignment, with consequent myo-fibril disorganization and proteolysis of the same by the ubiquitin-protessome system. Among some of the candidates for inducing the activation of these systems are glyco-corticoids, the inflammatory cytokines (interleukine-1 and TNF-a) and oxidative stress, which are associated to immobilization-induced atrophy[24,27].

Once the immobilization leads to significant muscular alterations, several authors had an interest to study the effects of exercise programs after this procedure[3,28-30]. The results they found deserve attention, because, although some authors show that exercise program are capable of reversing these alterations[3,29], there are also reports that the mechanical loading imposed on the atrophied muscle may lead to transient muscular injuries, with impairment of the maximal isometric strength[28,30]. These contradictory results may be related to the different methodological procedures used in these studies, such as different times of movement restriction imposed to the muscle (between seven days and four weeks), different techniques of muscle load removing (immobilization *versus* limb suspension), and also different load intensities imposed on the atrophied muscle after immobilization (free mobilization, isokinetic exercise, or walking belt). The results obtained in these studies suggest that mechanical loading is an important factor for the rehabilitation process; however, the quantity of loading to be used still remains undetermined.

Although the results from the present study show important short-term alterations on the muscle's morphometric and mechanical features, it was not possible to determine which factors directly influenced the decrease of the muscular resistance to traction. In this sense, further investigations, correlating sarcomere adaptations, muscle morphometry and mechanics, are necessary for the better understanding of immobilization affects on the skeletal muscle.

FINAL CONSIDERATIONS

The importance of this study is that it showed that only one week of immobilization was enough to yield significant muscular alterations, suggesting, therefore, the need for rehabilitation of muscles submitted to short-term immobilization. The results found, with an emphasis on muscular mechanics and structure, a basis for the selection of therapeutic treatments to be used after immobilization.

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