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Morphological effects of resumption of loading after immobilization of skeletal muscles in lengthened position in female rats

Efeitos morfológicos do retorno da sobrecarga após imobilização em alongamento de músculo esquelético de ratas

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Abstract

Background: In rehabilitation, immobilization of skeletal muscles in the elongated position is performed as a countermeasure in order to reverse the effects of severe muscle shortening and postoperative events. The return to normal functional activities is believed to stimulate mechanotransducers capable of reorganizing the normal muscle cytoarchitecture, but few data describing the histopathological changes relating to these procedures are available in the literature. **Objectives:** To assess and quantify histological abnormalities induced by immobilization of the extensor digitorum longus (EDL) muscle in elongation and to compare them with free movement of the animal after this procedure. **Methods:** Eighteen female Wistar rats were used, divided into the following groups: Control; Immobilized in plantar flexion (EDL in an elongated position) for 14 days (GI); Immobilized for 14 days and released for 10 days (GIL). EDL fragments were frozen, sectioned and processed through immunohistochemical reactions for collagens I and III and histochemical methods for myofibrillar adenosine triphosphatase using hematoxylin-eosin. **Results:** GI animals presented slight increases in collagen I and fiber expression in a degenerative/necrotic process, and reductions in the proportion of FT2A fibers and in the diameters of all fiber types, compared with the controls. In GIL, the quantity of collagen I returned to control conditions; the proportion of FT2D decreased; the number of centralized nuclei increased; and the fiber diameter was smaller than in GI. However, FT2B and FT2D expression did not reach the reference values. **Conclusions:** The data presented show that the recovery of function over a 10-day period was partially efficient with regard to recuperation of the characteristics of the EDL muscle after the period of immobilization. If the data are extrapolated to physiotherapeutic clinical practice, use of procedures directed towards primary dysfunctions of the muscle may favor a morphofunctional response in the segment and its full recovery.

Key words: skeletal muscle; immobilization; morphology; collagen; muscle fibers.

Resumo

Contextualização: Na reabilitação, a imobilização em alongamento do músculo esquelético é realizada como contramedida para reverter efeitos de encurtamento muscular severo e em eventos pós-cirúrgicos. Acredita-se que o retorno às atividades funcionais normais estimule mecanotransdutores capazes de reorganizar a citoarquitetura normal muscular, porém a descrição das alterações histopatológicas relacionadas a esses procedimentos são escassas na literatura. **Objetivos:** Avaliar e quantificar anomalias histológicas induzidas pela imobilização em alongamento do músculo EDL *(Extensor Digitorum Longus)* e confrontá-las com a livre movimentação do animal após esse procedimento. **Métodos:** Foram utilizadas 18 ratas Wistar, distribuídas nos grupos: controle (GC); imobilizadas em flexão plantar (EDL em posição alongada) por 14 dias (GI); imobilizadas por 14 dias e liberadas por dez dias (GIL). Fragmentos do EDL foram congelados, seccionados e processados com reações imuno-histoquímica para colágenos I e III e histoquímica para Adenosina Trifosfatase Miofibrilar e Hematoxilina-Eosina. **Resultados:** Os animais do GI apresentaram discreto aumento da expressão de colágeno I e de fibras em processo degenerativo/necrótico, redução da proporção de fibras tipo (FT) 2A e do diâmetro menor de todos os tipos de fibras, quando comparados com os animais do GC. Para o GIL, observou-se retorno da quantidade de colágeno I às condições controle, além de redução na proporção de FT2D, aumento do número de núcleos centralizados e do diâmetro menor das fibras quando comparadas com o GI, porém a expressão de FT2B e FT2D não atingiu os valores de referência. **Conclusões:** Os dados apresentados mostram que a retomada da função durante dez dias foi parcialmente eficiente na recuperação das características do músculo EDL após o período de imobilização e que, se extrapolados os dados à clínica fisioterapêutica, a adoção de procedimentos orientados às disfunções primárias do músculo pode favorecer a resposta morfofuncional do segmento e o seu íntegro restabelecimento.

Palavras-chave: músculo esquelético; imobilização; morfologia; colágeno; fibras musculares.

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Introduction

In rehabilitation clinical practice, muscle stretching is maintained as a countermeasure to reverse the effects of severe muscle shortening, such as in the progressive elongation with plaster that is undertaken after administration of botulinum toxin[1,2] and in cases of bone distraction to promote increases in limb length[3,4]. In addition, immobilization in the lengthened position is administered after surgery for muscle elongation[5] or in cases in which tendon rupture occurs in the contralateral muscle group[6,7].

Experiments on animals have demonstrated that immobilization in the lengthened position causes increased protein synthesis, reduction of the capacity to generate strength and increased quantities of connective tissue[8,9]. They have also shown that this procedure causes less severe muscle atrophy, together with lower loss of elastic properties, than do procedures involving immobilization in a shortening position[10]. Moreover, it promotes an increase of nearly 17% in the number of sarcomeres in series[11,12]. Concerning the muscle composition of the different types of myosin heavy chain (MHC), studies have produced contradictory findings. In fast-contraction muscles, some authors have identified an increase in the proportion of slow fibers, with a transition from the expression of fast isoforms of MHC towards slow ones[11,13], while others have observed a transition from faster fibers to faster ones[12,14]. However, only a few isolated reports on the histopathological and morphometric abnormalities that are induced by sustained elongation exist in the literature, thereby making it difficult to understand the complex mechanism of organization of the costamere and mechanotransducer proteins in cells.

Release followed by a period of disuse induces injuries to muscle fibers[15,16]. The loading imposed on muscles after a period of inactivity can cause damage to the sarcolemma and to the protein mesh of the costamere[17]. Changes to these structural proteins activate mechanoreceptors that, through a cascade of events, modify gene expression so as to favor protein synthesis[18,19]. This translates into increased collagen expression and synthesis[20], satellite cell activation[19] and increased production of contractile proteins[21] and signaling proteins[22,23]. These are the most important events relating to the resumption of loading activities after immobilization. These events together favor recovery of muscle volume and organization of the structural proteins, thereby reestablishing the biomechanical and functional characteristics. Recently, some studies have evaluated the changes imposed through resumption of loading after immobilization, but few of them have evaluated these changes in muscles that are subjected to continuous immobilization in elongation[11]. Therefore, this study had the goals of evaluating and quantifying the histopathological changes induced by release from a containment system after two weeks of immobilization of the extensor digitorum longus (EDL) muscle in an elongated position, in female rats.

Methods

Animals

Eighteen young adult female Wistar rats (*Rattus norvegicus albinus)* with a mean body mass of 200±30 g were kept in plastic cages in a controlled environment at a temperature of 24°C and 12-hour light and dark periods, with free access to standard food and water, at the vivarium of the Bioengineering Laboratory of Ribeirão Preto Medical School (FMRP), Universidade de São Paulo (USP), Ribeirão Preto, SP, Brazil. This study was approved by the Ethics Committee for Animal Research (protocol no. 06.1.692.53.8) of the Ribeirão Preto campus of USP.

The animals were randomly distributed into three groups of six animals: controls, which remained in plastic cages for a period of 14 days in order to be subjected to the same containment period as experienced by the other groups; immobilized group (GI), which remained with the right hind limb immobilized in ankle plantar flexion for a period of 14 consecutive days; immobilized and released group (GIL), which had their right hind limb immobilized in plantar flexion for a period of 14 days and were then released into the cages for a period of ten consecutive days.

Subsequently, they were sacrificed followed by extraction of the EDL muscle. The central portion of the dissected muscle was obtained, immersed in powder and subjected to fast freezing in liquid nitrogen. The fragments were stored in a freezer at -70°C until the time of sample processing.

Immobilization techniques

The right hind limb was immobilized after administration of an intramuscular anesthetic, using a mixture of ketamine hydrochloride (80 mg/kg) and xylazine hydrochloride (15 mg/kg), at a dose of 0.05 mg/100 g of the animal's body weight. A plaster device was then created to include the pelvis, hip and knee in total extension and the ankle in plantar flexion, in order to promote stretching of the EDL muscle (for further details, consult Mattiello-Sverzut et al.[16]).

Immunohistochemical reaction for collagen

The technique used in the Neuropathology Laboratory of the Department of Pathology, FMRP-USP, consisted of administration of anti-collagen type I monoclonal antibodies (clone COL-1,

Sigma) and anti-collagen type III monoclonal antibodies (clone FH-7A, Sigma) in order to characterize collagen types I and III, at dilutions of 1:3000 and 1:2000, respectively. The different antibodies were fixed on different slides on which muscle fragments of thickness 5 μm were mounted. The slides were then fixed in iced acetone for 20 minutes, washed in phosphate-buffered saline (PBS) and incubated with anti-collagen type I monoclonal antibody and anti-collagen type III monoclonal antibody at 4°C overnight. On the next day, the slides were washed and incubated using the NovoLink™ Max Polymer kit (Novocastra), which was composed of a post-primary polymer and a conjugated polymer. Firstly, the post-primary polymer was administered, and the muscle slices were left to incubate for 10 minutes at room temperature. Washing and incubation with conjugated polymer were then performed for another 10 minutes at room temperature. After this procedure, the samples were washed again in order to incubate them with diaminobenzidine (DAB) for 15 minutes. Subsequently, the samples were washed with distillated washed, counterstained with hematoxylin for one minute, dehydrated, diaphanized and, finally, mounted in Permount (Fisher). This protocol resulted in myonuclei and membrane proteins that were stained blue and connective tissue fibers that were stained brown. The different collagen types of the EDL muscle were assessed semi-quantitatively, following the protocol described by Kurose et al.[24], which consisted of immunostaining of collagen types I and III performed by three independent examiners. For the analysis, the following reactivity classification was used: (-) negative; (±) slightly positive; (+) weakly positive; (++) moderately positive; (+++) strongly positive.

Morphological analysis

The slides were analyzed quantitatively under an optical microscope (Leica DM 2500) by two examiners. Generic morphological characteristics were evaluated and the anatomopathological changes to the muscle tissues of each animal were accounted for through the hematoxylin-eosin reaction.

Morphometric analysis

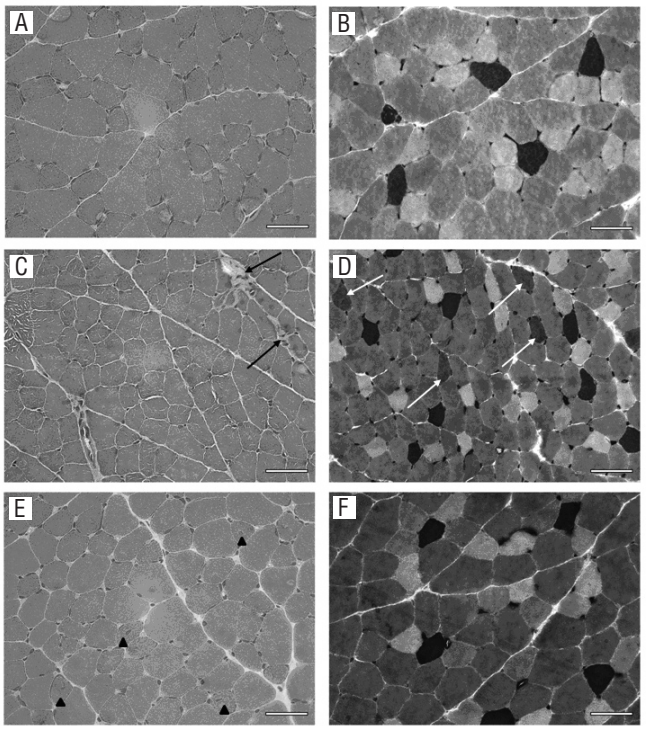
Using the QualiView software (Atonus), three random fields of the muscle fragment of each animal were photomicrographed, processed using the mATPase reaction[25] in pre-incubation pH 4.6 and compared with muscle sections processed at pH 4.3 and 9.4. The different types of fibers present in the EDL muscle were counted on each image, and the smallest diameter of each fiber was measured. Fibers that were incomplete on the images were excluded from the analysis. The measurement of the smallest diameter was selected to represent the cross-sectional area, in order to eliminate possible distortions in fiber area caused by oblique sectioning of the sample.

Statistical analysis

The statistical analysis on the smallest diameter and proportions of EDL muscle fibers in each animal was performed between the groups using the linear mixed-effect model, with a significance level of 5% (α=5%) and a confidence interval of 95% (CI=95%), using the PROC MIXED routine of the SAS software, version 9.2.

Results

The fragments of the EDL muscles of the control group animals presented polyhedral fibers, peripheral nuclei and variations in fiber size (Figure 1A). The immobilization caused a slight increase in the number of fibers undergoing degenerative/necrotic processes (Figure 1C). The GIL animals (Figure 1D and 1E) presented increased quantities of centralized nuclei compared with the controls (Figure 1A). Table 1 presents the mean and standard deviation values of the anatomopathological findings from the animals of the different groups analyzed. It



Control group (A and B), immobilized group (C and D), immobilized and released group (E and F) (bar: 50 μm). Note the following: (A) variation in fiber size and (B) predominance of fast-twitch type fibers and rarefaction of type 1 fibers (dark fibers); (C) fibers undergoing degenerative/necrotic process (dark arrow) and (D) presence of fibers with intermediated color (light arrow); (E) nuclear centralization (arrow head); and (F) predominance of fast-twitch fibers and rarefaction of type 1 fibers (dark fibers).

Figure 1. Photomicrographs of the EDL muscle. HE (A, C and E); mATPase pH 4.6 (B, D and F).

can be seen that, in absolute values, the nuclear centralization in the GIL animals and the degenerative/necrotic processes in the GI animals were the features with greatest modification in relation to the control group findings.

The assessment on the intramuscular connective tissue of the EDL muscle showed that there was a slight increase in the quantity of collagen type I in the GI animals and a slight reduction in the quantity of collagen type III in the GIL animals, as shown in Table 2.

Table 1. Histopathological changes to fibers [mean (± DP)] in the EDL muscle in the different groups analyzed.

	GC	GI	GIL
Nuclear centralization	1.3 (±1)	2.8 (±2.3)	6.3 (±7)
Lobulated fibers	0.5 (±1.2)	0.2 (±0.4)	0.5 (±0.5)
Degeneration/necrosis	3 (±1.7)	4.2 (±1.9)	1.3 (±0.8)
Regeneration	0.2 (±0.4)	0.2 (±0.4)	0
Splitting	1.2 (±1.6)	0.3 (±0.8)	1 (±0.9)

GC=control group; GI=immobilized group; GIL=immobilized and released group.

Table 2. Semi-quantitative assessment of interstitial collagen in the EDL muscle.

	GC	GI	GIL
Collagen I	±/+	+	±/+
Collagen III	++	++	+/++

GC=control group; GI=immobilized group; GIL=immobilized and released group; - negative; ± slightly positive; + weakly positive; ++ moderately positive; +++ strongly positive.

The analysis of the proportions of the different types of fibers in the EDL muscle indicated that immobilization caused a reduction only in the number of FT2A fibers (GI vs. controls, $p<0.001$), while release into the cage after the immobilization period reduced the number of FT2D fibers (GIL vs. controls, $p<0.01$; GIL vs. GI, $p<0.0001$), in relation to the control and GI animals (Figure 2). The data on the smallest fiber diameters showed that immobilization caused a reduction in the smallest size of all fiber types in relation to the respective control values (GI vs. controls GC, $p\leq0.03$) (Table 2). On the other hand, release of the animals into the cage caused an increase in the diameter values of FT1, FT2A, FT2AD, FT2D and FT2B, compared with those of the GI animals (GIL vs. GI, $p<0.01$) (Table 3).

Discussion

In rehabilitation clinical practice, there are still some questions with regard to the readaptation of some variables of muscle tissue that is subjected to procedures involving disuse and subsequent release for activities of daily living. Efficient indication of specific therapeutic approaches or decisions not to indicate certain procedures can be discussed in relation to several factors. Among these are the type of disuse, the period of movement restraint, the contractile characteristics of the muscle (or muscle group) damaged and the morphological findings. In this respect, the present experimental study conducted

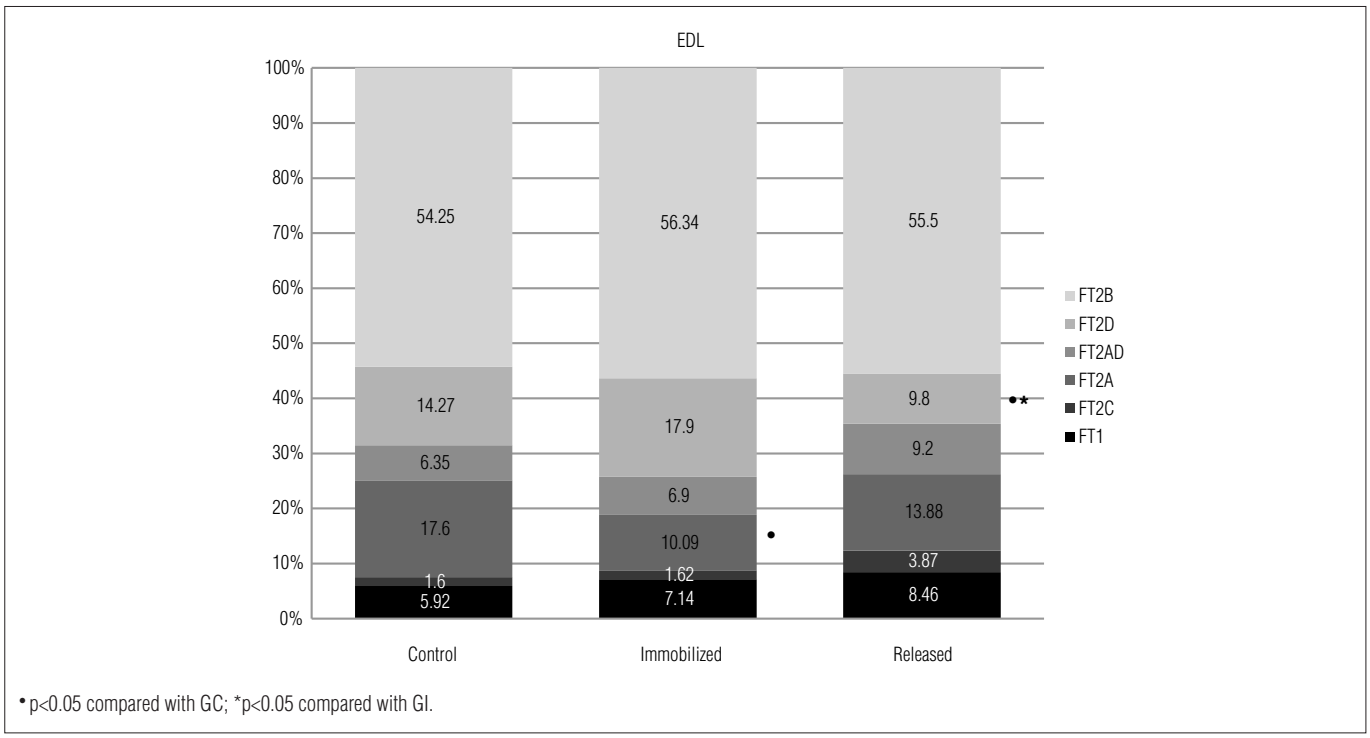


Figure 2. Percentages of different types of fibers in the EDL muscle in the groups analyzed.

on female Wistar rats found that 14 days of immobilization of the EDL muscle in elongation and subsequent release of the animals into the cage for another 10 days gave rise to significant changes to the expression of interstitial collagen and the proportions and tropism of muscle fibers.

Effects of immobilization in elongation

Specifically, the procedure of immobilization of the EDL muscle, in the elongated position for 14 days, caused atrophy of the different types of muscle fibers, reduction only in the proportion of FT2A and a slight increase in the quantity of collagen I. Immobilization in an elongated position is considered to stimulate longitudinal tension, but it does not cause overload on skeletal muscle. In the other hand, it may induce modifications to factors relating to myogenic regulation. A study conducted by Gomes et al.[27] found that daily sessions of stretching of the soleus of normal rats that were maintained for 30 minutes a day for 7 days only modified the expression of mRNA to atrogin-1, and did not change the expression of mRNA to MyoD and myostatin. According to Kim et al.[28], there is a relationship between the expression of atrogin and ubiquitin-ligase, which plays an important role in the process of protein degradation and, consequently, in the regulation of muscle mass. In parallel, studies conducted by Williams et al.[29] and Ahtikoski et al.[30] found that immobilization in an elongated position for a week did not cause any change to the amount of connective tissue. In the second of these studies, it was found that keeping a muscle in elongation prevented reduction of the mRNA levels for collagen I. In addition, the mRNA concentrations for type I and III collagens showed reductions on the third day of immobilization of the tibialis anterior, but returned to the control values after the seventh day[31]. On the other hand, Jósza et al.[11] found that the quantity of connective perimysial tissue in the soleus and gastrocnemius muscles of animals subjected to immobilization in dorsiflexion increased during the first week post-procedure, but progressed to five times this amount after the third week. The data highlighted above suggest that the duration of joint restriction is an important variable relating to occurrences of changes to the collagen and conjunctive tissue, like those identified in the present study. In the extracellular matrix, collagen type I determines tensile strength and stiffness and therefore, from a biomechanical viewpoint, reduction of the level of this protein can impair the capacity to support loading in the elastic phase[32,33], which occurs before the rupture, thus making the tissue more vulnerable to longitudinal stress.

Immobilization of the fast-twitch type fibers in elongation can cause an increase in the fraction of fast fibers, thereby leading to a transition from fast fibers to fibers that are even faster[12]. Since the EDL is mainly composed of fast-twitch fibers, it could be seen that after the period of immobilization of this muscle, there was a significant reduction in FT2A, although without statistical increases in the other fiber types. Therefore, it is believed that the duration of the immobilization used in the present study was insufficient to confirm the direction of the transition that was induced by the intervention to maintain stretching, as highlighted by previous authors.

Table 3. Mean smallest diameter (μm) with respective confidence interval for the different fiber types in the EDL muscle.

	GC	GI	GIL
FT1	24.16 (22.25 - 26.07)	20.80• (18.94 - 22.66)	24.31* (22.41 - 26.20)
FT2C	23.89 (21.07 - 26.71)	20.19• (17.45 - 22.92)	22.77 (20.58 - 24.97)
FT2A	23.72 (22.04 - 25.40)	20.87• (19.10 - 22.65)	23.22* (21.41 - 25.02)
FT2AD	24.59 (22.70 - 26.47)	21.49• (19.57 - 23.39)	23.64* (21.75 - 25.53)
FT2D	29.95 (28.24 - 31.66)	23.37• (21.70 - 25.05)	28.46•* (26.57 - 30.35)
FT2B	32.65 (31.05 - 34.23)	23.12• (21.54 - 24.71)	31.48•* (29.88 - 33.08)

GC=control group; GI=immobilized group; GIL=immobilized and released group; • $p<0.05$ compared with GC; * $p<0.05$ compared with GI.

The average value of the smallest diameter of the animals' fibers immobilized in elongation was smaller than that of the control animals, as also observed by Chopard, Pons and Marini[34], Stelzer and Widrick[35] and Gehrke et al.[36] On the other hand, Yang et al.[37] immobilized the EDL muscle in an elongated position for six days and found that there were increases in muscle mass and in local expression of IGF-1. A study developed by De Deyne et al.[3] showed that the changes to IGF-1 may contribute little towards the transformations undergone by the EDL immobilized in an elongated position. Hence, it is possible that the duration of immobilization was an important factor, or that this protein has other roles, i.e. not only for promotion of muscle growth. Although the methodology used in the present study did not allow investigation of ultrastructural alterations and proteins specific to the costamere, the presence of fibers undergoing a process of degeneration/necrosis and others with nuclear centralization makes it possible to suggest that damage to the sarcolemma and subsequent architectural modification to the proteins of the costamere occurred. This is of fundamental importance with regard to the stability of the sarcolemma and nucleus positioning[38], especially for the EDL muscle, as identified by O'Neill et al.[39]. Destructuring of the sarcolemma and the proteins associated with it may, in turn, have evoked a cascade relating to lysosomal proteolysis via cathepsin and ATP-dependent ubiquitin proteasome[40,41]. In addition, titin, a structural protein of importance for maintaining the structure of the sarcomeres, may have been damaged by the intensity of the elongation developed during immobilization. The

elongation may have exceeded the functional amplitude of the strain of the PEVK domain (proline-glutamine-valine-lysine-rich domain), thus causing disorganization of the contractile myofibrils and, consequently, signaling fiber degeneration[42,43]. In parallel with the disarrangement of the sarcolemma, tissue repair and regeneration was highlighted by nuclear centralization and the slight increase in type I collagen. Summarizing, it should be borne in mind that immobilization promotes a reduction in protein synthesis and an acceleration in protein degradation. However, the levels of atrophy and modification to collagen synthesis seem to be different for each muscle, depending on the type of fiber composing the muscle[30] and the position in which the muscle remained immobilized[35].

Effects of the lengthening position on post-immobilization release

Free movement among the animals was able to restore the diameter ratio to the proportions seen in the control group, for almost all types of fibers except FT2B and FT2D. Since the EDL muscle is mainly composed of fast-twitch fibers, which are the first to be recruited, the return to activity may have caused greater damage to these fibers during the initial phase of release[44], thereby delaying their recovery process and volume gain. Studies conducted by our research group have shown that after immobilization of the plantar muscle (which is also composed predominantly of fast-twitch fibers) in a shortened positions for ten days, its morphological characteristics are recovered after ten days of rehabilitation, either by stretching or by eccentric training (Cornachione et al.[45], unpublished results). Furthermore, we observed a slight reduction in the expression of collagen III.

This type of collagen is responsible for compliance of the extracellular matrix, thereby allowing greater mobility between the muscle fibers. As observed by other authors on the seventh day post-immobilization in an elongated position[30,31], the present study also showed that the collagen type III content did not differ from what was observed in the control group on the fourteenth day after immobilization. Although there are no reports in the literature on mRNA and/or pre-collagen type III expression after restraint removal using the methodology of the present study, it can be inferred that the release from restraint favored reorganization of these macromolecular structures, as suggested by Coutinho, DeLuca and Salvini[46]. Furthermore, in muscles with a predominance of glycolytic fibers, exercise loading increases the expression levels of mRNA and of metalloproteinase 2 (MMP-2) itself[47]. This, in turn, besides acting to degrade the connective tissue of the extracellular matrix, increases the activation of satellite cells[48], thereby assisting in the process of tissue regeneration[49]. This is shown by the increased presence of centralized nuclei, as found in the present study. In parallel, the increase in the smallest diameter of most of the fibers can be explained in terms of the activation of a cascade of events involving growth factors and muscle regeneration, such as IGF-1 and MGF[27,50].

Considering the variables analyzed and the methodology used, it can be concluded that immobilization in the lengthened position for 14 consecutive days caused histopathological changes in the EDL muscle that were consistent with disuse, featuring atrophy of all kinds of fibers and increased levels of collagen I. The return of loading for ten days, with the release of the animal, generated mechanical stimuli that were capable of promoting activation of mechanisms that were responsible for retrieving the patterns of the EDL muscle, except for the tropism and the proportion of fast-twitch fibers. From the results obtained in this study on female rats, it is hypothesized that some care should be taken in clinical rehabilitation when subjecting segments to loading after brief periods of immobilization in the lengthened position.

It is important to highlight the fragility of the cellular and interstitial components to longitudinal stress, as well as the likely functional muscle weakness characterized by atrophy and reduced proportions of fast fibers. Therefore, it is suggested that the recovery of function should be gradual based on the use of exercises that can increase the activity of oxidative and glycolytic fibers (FT2A), which suffered a reduction in number and volume. One parallel action would be to use electrical stimulation of mean frequency[51] in the muscle group that is kept stretched through immobilization, which would help in the specific recovery of these fibers and, thus, in restoring muscle strength and function.

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