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ORIGINAL ARTICLE

USE OF PASSIVE JOINT MOBILIZATION AND THERAPEUTIC ULTRASOUND IN THE REMOBILIZATION OF THE EXTENSOR DIGITORUM LONGUS MUSCLE IN WISTAR RATS

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ABSTRACT

Objective: The aim of the study was to analyze the tissue repercussions of passive mobilization and therapeutic ultrasound, in isolation and in combination, on the morphology of the extensor digitorum longus (EDL) muscle of Wistar rats in an immobilization model.

Materials and methods: 40 rats were used, divided into 5 groups (n = 8): immobilization group (IM), free remobilization group (FR), remobilization and passive mobilization group (PM), remobilization group treated with therapeutic ultrasound (US) and remobilization group treated with passive mobilization and therapeutic ultrasound - association (PM+US). The animals were immobilized in the stretching position with a plaster bandage on the right pelvic limb for 21 days and distributed according to treatment for 3 weeks, while IM was euthanized after the immobilization period. After collection, the EDL muscle was processed for general morphology analysis. Statistical data was analysed using SPSS 20.0® software using generalized linear models ($p < 0.05$).

Results: Stretching immobilization for 21 days caused significant tissue alterations to the EDL, as evidenced by amorphous muscle fibres and atrophy, intense inflammatory infiltrate and various stages of necrosis, as well as alterations to the muscle spindles and connective tissue hypertrophy. The groups that received the therapies alone showed lower degrees of fiber damage, standard muscle spindles and a reduction in connective tissue. However, the association group obtained the best morphological results.

Conclusion: Treatment with passive mobilization and therapeutic ultrasound is promising in reducing muscle tissue damage, but associated therapies accelerate the process of regeneration of EDL muscle tissue after immobilization in stretching.

KEYWORDS

Muscle atrophy, immobilization, early mobilization, ultrasound therapy

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INTRODUCTION

Skeletal striated muscle tissue is capable of adapting, both structurally and functionally, in response to various stimuli, and immobilization has significant deleterious effects[1,2]. In this sense, when the immobilization of a body segment is necessary, as in orthopedic disorders, it can cause negative repercussions on any component of this tissue, such as joint stiffness, muscle hypotrophy, connective tissue proliferation, reduced extensibility, decreased strength, endurance and muscle glycogen reserve, affecting its functionality[3–5].

It is known that deleterious adaptations are influenced by the position in which the muscle is stabilized. Animal experiments have shown that when the muscle is elongated, there are fewer tissue adaptations when compared to immobilization protocols in a shortened position[6]. In addition, these modifications changes in relation to the muscle analyzed, due to the type of fiber, with those with an oxidative predominance being more affected than those with a glycolytic predominance[7].

It is a fact that the longer the limb remains immobilized, the greater the deleterious repercussions on skeletal muscle tissue[3,8], even short periods of immobilization are enough to cause significant tissue adaptations[9]. Thus, there is a need to look for therapeutic strategies aimed at rehabilitation in order to speed up muscle remobilization after immobilization, with the aim of promoting a return to functionality and quality of life for individuals after immobilization. In this sense, physiotherapeutic treatment offers easily accessible and low-cost tools, such as manual therapy and electrothermophototherapy[10,11].

Among the manual therapy modalities, passive mobilization, with techniques such as Maitland mobilization, has benefits that are not only related to the musculoskeletal system, but also to emotional and social aspects, with the hypothesis that it acts on the cardiovascular, respiratory, neurological and emotional systems[12,13]. Specifically in muscle tissue, a reduction in muscle fiber atrophy and general tissue improvement resulting from immobilization using passive joint movement have already been observed[14].

Therapeutic ultrasound is another modality that can be used in a variety of treatments in different environments, such as hospitals. It works on ligament injuries, tendinopathies, among others. It is a resource that involves a form of mechanical energy, at low intensities, being transmitted to the tissues at high frequency, promoting changes in the intra and extracellular environment related to tissue regeneration, increased metabolism and local circulation, as well as increased extensibility and collagen production[15–17].

However, there are few studies demonstrating the effects of using these therapies alone or in combination on muscle tissue that remains in an elongated position during prolonged immobilization, such as the extensor digitorum longus (EDL) muscle. Therefore, it is promising to evaluate the repercussions that both therapies have on the locomotor system, aiding evidence-based clinical practice. Therefore, the aim of this study was to analyze the tissue repercussions of passive mobilization and therapeutic ultrasound alone and in combination, on the morphology of the EDL of Wistar rats immobilized in plantiflexion, analyzing the level of destructuring in the muscle, the characteristics of the muscle spindles and the intramuscular connective tissue.

MATERIALS AND METHODS

Animals and experimental groups

This is an experimental, randomized and exploratory study, approved by the Ethics Committee on Animal Use (CEUA) of the Western Paraná State University (UNIOESTE) (Protocol no. 09-21) on the 12/07/2021 and carried out in compliance with the ethical precepts of animal experimentation.

Forty 10-week-old Wistar rats weighing an average of 278 ± 28 g were obtained from UNIOESTE's central animal house and placed in polypropylene plastic boxes at 22°C room temperature, with a 12-hour light/dark photoperiod and access to water and food ad libitum.

The animals were randomized into 5 experimental groups, with 8 animals in each group: immobilization group (IM), free remobilization group (FR), remobilization and passive mobilization group (PM), remobilization group treated with therapeutic ultrasound (US) and remobilization group treated with passive mobilization and therapeutic ultrasound - association (PM+US). After the adaptation phase (seven days), the animals were immobilized with a cast orthosis with a plaster bandage, in which they remained for 21 consecutive days. At the end of this period, the orthosis was removed and the IM animals were euthanized. The other groups underwent remobilization, with FR remaining freely mobile in the breeding box without any intervention, PM being treated with passive mobilization, US with therapeutic ultrasound and US+PM with a combination of techniques.

Each group received its own specific protocol over the course of the 3-week experiment, with 3 therapy sessions being held each week, totaling 9 sessions at the end of the intervention. After 24 hours of the last therapy, these groups were euthanized, with EDL collection.

Immobilization and remobilization protocol

The protocol used for immobilization was adapted[18,19], The animals were anesthetized (ketamine hydrochloride: 95 mg/kg; xylazine hydrochloride: 12 mg/kg, intraperitoneally) and immobilized with a cast orthosis for 21 consecutive days. The orthosis was molded from the abdominal region, just below the costal gradient, and continued to the right pelvic limb. The hip and knee joints remained in full extension and the tibiotarsal joint in maximum plantar flexion, with the EDL muscle remaining in a stretched position.

For passive joint mobilization, the protocol was applied to

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the right pelvic limb of the animals belonging to the PM and US+PM, in which grade IV passive joint mobilization was performed (small amplitude mobilization that reaches the restrictive barrier). In the knee joint, the passive movement was in the posteroanterior plane; and in the ankle, anteroposterior. For both ipsilateral joints, there were approximately 50 movements in one minute, three times a week, for 3 weeks, totaling 9 sessions[20].

For the ultrasound therapy, the Sonoplus Ibramed® device was used, with a calibration certificate for the duration of the study. The parameters used were: frequency of 1.0 MHz, with an ERA of 1 cm^2, continuous emission, intensity of 0.5 w/cm^2 for 2 minutes on the knee (1 minute on the lateral side and 1 minute on the medial side) and 2 minutes on the ankle (1 minute on the lateral side and 1 minute on the medial side) of the right pelvic limb of the animals belonging to the US and US+PM groups. Sessions were also held 3 times a week for 3 weeks, totaling 9 sessions.

Euthanasia of the animals and histomorphological analysis of the EDL

The IM animals were euthanized immediately after removal of the orthosis after 21 days of immobilization, while the FR, PM, US and US+PM were euthanized after 21 days of remobilization with the protocols. For euthanasia, the animals were previously weighed to adjust the anesthetic dose and submitted to an intraperitoneal overdose of ketamine hydrochloride and xylazine. The EDL was dissected, fixed in Metacarn (70% methanol + 20% chloroform + 10% glacial acetic acid) for 24 hours and stored in 70% alcohol until processing. To make the slides, they were dehydrated in increasing series of alcohols, diaphanized in n-butyl alcohol and embedded in histological paraffin. Subsequently, the muscles were sectioned into 5 µm thick cross-sections using a microtome (CUT 4055 Olympus®) and stained in HE for general analysis of the muscle tissue.

The slides were analyzed under light microscopy using a 40× objective, and the morphological characteristics observed were classified and presented in the form of a morphological chart.

Analysis of connective tissue and muscle spindles

To estimate the density of connective tissue and check the characteristics of the neuromuscular spindles, five animals were selected from each group and the same processing was carried out as for HE, only with specific Masson's Trichrome staining for connective tissue. The slides were analyzed under a light microscope (BX60 Olympus®), and 10 photomicrographs were taken of each animal under a 40× objective and analyzed using the GIMP 2.10.36 program. The relative area of the connective tissue (area density) was calculated by dividing the total pixels of the photomicrograph by the total pixels of the connective tissue marking[19].

To analyze the muscle spindle, the slides were photomicrographed under a light microscope (BX60 Olympus®, Tokyo, Japan) at a 40× objective and analyzed for area, capsule thickness and number of intrafusal fibers using Image-Pro-Plus 6.0 (Media Cybernetics®, Silver Spring, USA)[21]. The data collected was organized in a Microsoft Excel® table to obtain the averages for each animal.

Statistical Analysis

SPSS 20.0® was used for statistical analysis of the histopathological index, connective tissue density and muscle spindle characteristics. Comparisons were made using Generalized Linear Models, with Fisher's post-test (LSD). The significance level adopted was $p < 0.05$ and the results are expressed as mean and standard deviation. Cohen's d effect size analysis (ES) was also used.

RESULTS

Qualitative analysis and histopathological index

Morphologically, the IM *(Fig. 1A)* showed characteristics of tissue damage, muscle fibers with signs of degeneration, some with an amorphous shape, atrophic, with dye differences and in various stages of necrosis, presence

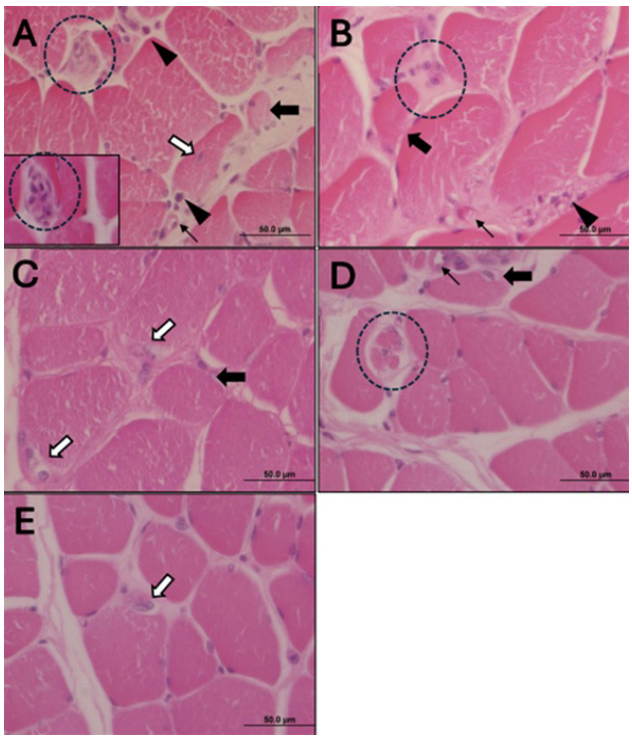


Figure 1. *Cross-sectional photomicrographs of the extensor digitorum longus muscle of Wistar rats showing the histology of the muscle tissue, stained with HE. Immobilized groups - IM (A), free remobilization - FR (B), passive remobilization - PM (C), remobilization with ultrasound - US (D), remobilization with association – PM+US (E). They show amorphous fibres with atrophy (black arrow), fibres with a difference in tone and in stages of necrosis (dotted circle), an inflammatory infiltrate (arrowhead), an internalized nucleus with a basophilic halo (white arrow), blood vessels (thin mushroom).*

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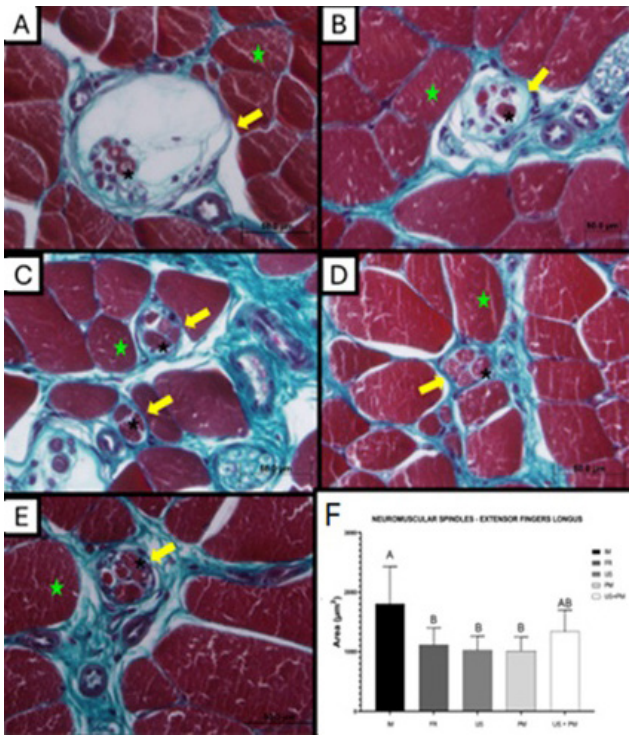


Figure 2. *Cross-sectional photomicrographs of the extensor digitorum longus muscle of Wistar rats showing the histology of the muscle tissue, stained with HE. Immobilized groups - IM (A), free remobilization - FR (B), passive remobilization - PM (C), remobilization with ultrasound - US (D), remobilization with association - US+PM (E). They show amorphous fibres with atrophy (black arrow), fibres with a difference in tone and in stages of necrosis (dotted circle), inflammatory infiltrate (arrowhead), internalized nucleus with basophilic halo (white arrow), blood vessels (thin arrow).*

of inflammatory infiltrate in the interstitium and performing phagocytosis. The nuclei were also centralized, with a basophilic halo and an apparent increase in the nuclear domain. The FR group *(Fig. 1B)* also showed some lesions, but less frequently than the IM group, such as tissue disorganization, amorphous and atrophic fibers with dye differences, inflammatory infiltrates and early stages of necrosis. In addition to angiogenesis, the blood vessels were congested.

The treatment groups showed tissue improvement compared to the group that had its remobilization free, in PM *(Fig. 1C)* and US *(Fig. 1D)* few rounded or amorphous and atrophic fibers were seen. Muscle fibers with a basophilic halo and an increase in the nuclear domain were seen. Angiogenesis and degenerating fibers were more frequent in US.

On the other hand, the group treated with the combination of passive mobilization and ultrasound, US+PM *(Figure 1E)*, showed few tissue changes, such as homogeneous muscle fibres, few blood vessels and no inflammatory infiltrate or muscle necrosis.

Muscle spindles

With regard to muscle spindles, it was possible to observe greater disorganization of the intrafusal fibers and the capsule in groups IM *(Fig. 2A)* and FR *(Fig. 2B)*, as well as a significant increase in area in IM. The PM, US and US+PM groups had spindles with standard characteristics and preserved morphology *(Fig. 2C-E)*.

In the statistical analysis, the only difference observed between the groups ($p < 0.05$) was in the area parameter, as shown in *Figure 3F*, in which IM had a larger area than the FR, PM and US groups. As for the US+PM group, there was no significant difference between the groups. With regard to effect size, for FR it was -4.158, for PM -4.804, for US -4.878 and for US+PM -2.660, i.e. with the exception of the remobilization group with the combination of ultrasound and passive mobilization, all the others had smaller spindles than the immobilized one.

There were no statistical differences in capsule thickness. However, the effect size showed 2.444 for FR, 0.556 for PM, 1.222 for US and -0.778 for US+PM, indicating that the free remobilization group had greater capsule thickness and the combined therapy group had less. There was also no significant difference between the groups in the number of intrafusal fibers.

And the effect size showed for FR -1.903, for PM -2.287, for US -1.069 and for US+PM 1.347, which may infer that the associated therapies group had a greater number of intrafusal fibers.

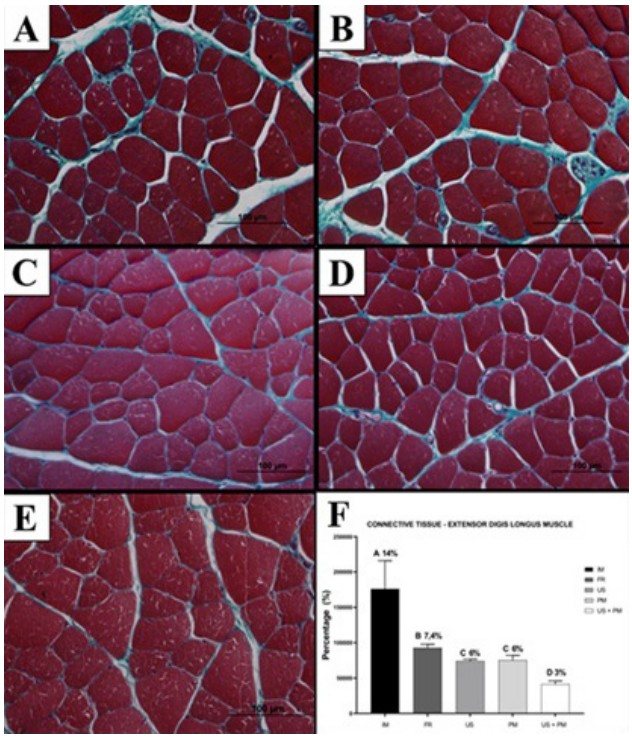


Figure 3. *Photomicrographs of the extensor digitorum longus muscle of Wistar rats in cross-section, showing connective tissue, stained with Masson's Trichrome, and graph of connective tissue density. Legend: Group IM (A), FR (B), GMP (C), US (D), US+PM (E). Muscle fibers are shown in red and pink, and connective tissue in green. Image F represents the graph of the amount of connective tissue based on the values from the statistical analysis.*

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Estimation of intramuscular connective tissue

Analysis of connective tissue density showed that IM *(Fig. 3A)* had the greatest amount of connective tissue in the endomysium and perimysium. Compared to the other groups, FR *(Fig. 3B)* showed an increase in connective tissue, although not as significant as in IM, followed by GMP *(Fig. 3C)* and US *(Fig. 3D)*. It is worth noting that US+PM *(Fig. 3E)* was the group that showed the least amount of connective tissue.

The statistical analysis ($p < 0.05$) showed a difference between the groups in the experiment, with IM having 14% connective tissue, FR 7.4%, PM and US 6%, showing that both groups did not differ significantly from each other, and US+PM 3%, which confirms the lower amount *(Fig. 3F)*. In terms of connective tissue morphology, the slides maintained a pattern of organization. The effect size was -10.594 for FR, -13.573 for PM, -13.407 for US and -18.522 for US+PM, again indicating that the least amount of connective tissue deposition occurred in the association group.

DISCUSSION

This study analyzed the tissue repercussions of joint immobilization for 21 days, as well as therapeutic interventions with passive joint mobilization, therapeutic ultrasound and a combination of both therapies in the remobilization of the EDL for 3 weeks. Immobilization reduces protein synthesis and increases tissue proteolysis, causing atrophy and hyaline degeneration culminating in muscle fibre necrosis, which often accompanies muscle atrophy in conditions of prolonged immobilization. Degeneration can be exacerbated by conditions such as malnutrition and lack of muscle contraction stimulus. These findings emphasize the need for therapeutic interventions to prevent muscle degeneration and promote recovery in individuals subjected to prolonged immobilization[6,7,22]. This corroborates the findings of the present study, since after the immobilization time, the IM showed intense muscle atrophy, with fibers in various stages of necrosis, angiogenesis and fibrosis, as evidenced by the significant increase in intramuscular connective tissue.

Particularly in relation to connective tissue, joint immobilization generates disorganization of contractile tissue, stimulating fibroblast proliferation, which in turn increases collagen synthesis and, consequently, local stiffness[23]. The current study showed similar results, as IM had a greater amount of connective tissue, followed by FR, with a significant decrease for the treated groups, especially for the combination of therapies. Passive mobilization generates physical stress on muscle and joint tissue, which may have been responsible for the reduction in the concentrations of collagen deposited[24,25]. Furthermore, therapeutic ultrasound has an important effect on tissue renewal, especially on collagen turnover[26], which may have contributed, in isolation or in association, to a regular and more organized deposition in the immobilized tissue.

Studies indicate that the muscle in the EDL is composed predominantly of type II fibers, which are fast twitch fibers. Specifically, one study revealed that the EDL contains around 75.7% type IIb fibers, which are fast twitch and fatigable fibers[27]. In addition to this, a more recent study also showed muscle characteristics with a predominance of type II fibers[28]. Type II muscle fibers tend to be more susceptible to morphological changes due to their metabolism and function, and are more prone to structural changes in response to different stimuli or conditions. This explains the changes found in the IM group, as well as the improvement in the treatment groups, demonstrating the high morphological plasticity in muscles with a predominance of this type of fiber.

In relation to the neuromuscular spindles, which are very important structures for proprioception, there was an increase in the area in the immobilization group compared to the other groups, as well as disorganization of the capsule and intrafusal fibers.

This finding can be observed in the notes by Kröger and Watkins[29], in which they mention changes in neuromuscular spindles in the condition of muscular dystrophy, a disease which, among other factors, causes muscular atrophy due to the degeneration of musculoskeletal tissue, a sign also observed in immobility syndrome. According to the authors, an analysis carried out on the muscle spindles of mice showed that the disease caused an increase in the diameter of the external capsule, which can be compared to the increase in the area of the spindles in the IM. However, in human studies, it was shown that despite the visible changes in the proprioceptive system, few morphological changes were observed, such as the thickness of the capsule and intrafusal fibers.

This suggests that the changes found are important and could affect muscle functionality. In the effect sizes, it was possible to observe that the greatest differences were found precisely in IM with regard to the increase in thickness. The FR showed a greater increase in capsule thickness, and finally there was a greater number of fibers for the combination of therapies. Bearing in mind these findings and the fact that neuromuscular spindles are sensory receptors that work together with the central nervous system (CNS), informing it of changes in muscle length, they are extremely important for performing coordinated movements, walking and maintaining a stable posture. From this, the CNS adjusts the movement to the appropriate angles, preventing injuries and promoting motor control for good functionality[29]. In other words, it's likely that an increase in the area and thickness of the capsule is not a good indicator of muscle functionality, unlike an increase in the number of fibers, which can lead to an increase in output and more information for the CNS to develop more efficient responses.

In the present study, the animals that were treated

with passive mobilization showed muscle fibres with a more regular morphology, a reduction in the amount of connective tissue, fewer blood vessels, as well as a better structural organization of the neuromuscular spindles, showing that passive mobilization had positive tissue repercussions for the remobilization of the EDL. The use of this therapy is therefore recommended, as it improves muscle tissue morphology, which can stimulate mechanoreceptors[28].

With regard to therapeutic ultrasound, which is widespread in physiotherapy, it is known that the mechanical energy produced comes from a deformation caused by an alternating current which, when flowing through a crystal housed in a transducer, alters it. When it reaches the tissues, it generates a series of changes through its mechanical effects: formation of new vessels with increased blood circulation, seen in the US group, tissue regeneration, soft tissue repair, protein synthesis, activation of the calcium cycle, reduction of muscle spasms and pain relief; in other words, its effects are capable of providing cell proliferation and tissue repair[16,26,30].

In this study, UST was applied in continuous mode, knowing that, at intensities such as the one proposed, the tissue temperature would increase by 2°C, obtaining a thermal effect and, consequently, changes in the viscoelastic properties of collagen and tissue extensibility, with an increase in local metabolism, which may have helped to restructure the spindle both in the area and thickness of the capsule, but may have been deleterious to the number of spindle fibers[31]. There was a significant presence of vascular turgidity, which suggests increased blood flow. This finding was common in the groups studied and is directly related to the increase in blood flow due to the thermal effect in the groups that used ultrasound (US and US+PM)[32].

In relation to the association, although there were no significant differences in the number of intrafusal fibers, it was possible to verify by the effect size that there was an increase in these for the association group, which suggests that such structural changes may improve functionality, as demonstrated by Kröger & Watkin[29].

In this sense, it was observed that the treatments alone accelerated the process of regeneration of the muscle tissue of the EDL after 3 weeks of immobilization compared to the group that did not receive treatment, FR, being amplified in the group that received the combination of both, which may have repercussions on the improvement of functionality. It is understood that, although analyzed with caution, these findings may contribute to future studies in humans, since a relationship has been observed between studies with rodents and humans in relation to musculoskeletal plasticity. Limitations include the absence of collagen type analysis and muscle fiber typing, as well as diversity in the dosimetry of both ultrasound and passive mobilization, which we suggest future studies should take into account.

CONCLUSION

In conclusion, the EDL immobilized in the stretching position showed significant and classic histomorphological changes described in immobility syndrome, and this study provides relevant information on the repercussions on connective tissue and muscle spindles that are affected by the type of immobilization. Treatment with passive mobilization and therapeutic ultrasound proved effective in reducing tissue damage, reorganizing neuromuscular spindles and reducing connective tissue. The combination of passive mobilization and therapeutic ultrasound treatments showed the best results in recovering the tissue damage caused by immobilization in the EDL muscle.

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