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Effect of Therapeutic Ultrasound on the Recovery of the Tibialis Anterior Muscle in Remobilized Wistar Rats after a Period of Immobilization: An Analysis Using the Pathological Index

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SUMMARY

Background. Prolonged joint immobilization causes muscle hypotrophy and joint stiffness, which worsen as the immobilization time increases. Muscle remobilization is essential to restore muscle mass and activity as soon as possible. There are various treatments for muscle remobilization, but therapeutic ultrasound has proved to be a promising technique, with thermal and athermic actions, generating possibilities for increasing tissue extensibility and cell metabolism, helping with the anabolic effects of static stretching.

Objective. To evaluate the histopathological changes in the tibialis anterior muscle of Wistar rats after remobilization using therapeutic ultrasound, using the histopathological index.

Methods. 21 male Wistar rats were used, divided into 3 groups (n = 7): immobilization group, immobilization and free remobilization group, immobilization and remobilization with ultrasound group. Immobilization was carried out with the knee flexed at 120° and total ankle plantiflexion for three weeks. Ultrasound was used at a frequency of 1.0 MHz with an intensity of 0.5 w/cm^2, lasting 2 minutes on the knee and a further 2 minutes on the ankle. Analyses were carried out using the histopathological index to measure the level of alterations and lesions in the muscle tissue, classifying them as severe, moderate or mild.

Conclusions. It was concluded that the anabolic stimulus of ultrasound was favorable to the trophic recovery of muscle tissue.

KEY WORDS

Immobilization; ultrasonic therapy; pathology; skeletal muscle; physical therapy modalities.

INTRODUCTION

The reduction in muscle activity, especially when there is a lack of mechanical loads, such as in cases of joint immobilization, leads to various changes and adaptations in muscle tissue, including a reduction in physical performance, with loss of mass, endurance and muscle strength, with adaptation in neural activity generating a reduction in motor control. In addition to these effects, there is also an increase in the amount of intramuscular fibrous and adipose tissue, an angiostatic effect and a reduction in the pain threshold, such characteristics being observed in experimental studies in both humans and animals (1-4).

Another effect that immobilization generates is inflammatory infiltration, which can be seen in degenerative changes in a joint immobilization model and joint contractures associated with fibrosis (1, 5-7). During remobilization, there are doubts about the behavior of the inflammatory process. It is argued that it tends to be less important than during immobilization (8), or that there is an increase in it due to microlesions and the involvement of nitric oxide (9).

Remobilization is essential for joint and muscle patterns to be restored (9-11). There are several techniques for the remobilization period, however, there is no unanimity as to the best form, nor regarding the necessary period of stimulation (4, 10, 12-17). One of these techniques is therapeutic ultrasound, which among its effects generates analgesic, anti-inflammatory, pro-repair and tissue regeneration action, in addition to improving vascularization, stimulating cell activity and protein synthesis, contributing to tissue reorganization (18-20).

With a view to the striated skeletal muscle, evaluations of infiltrates and hypercellularity have been analyzed in the literature (21). The changes caused by both immobilization and remobilization can be assessed quantitatively by a histopathological index proposed by Zazula *et al.* (22) which analyses different degrees of tissue damage, with good replicability and parsimony. In view of the gap in this type of analysis in remobilization aided by the effects of therapeutic ultrasound, the aim of this study was to evaluate the histopathological changes in the tibialis anterior muscle of Wistar rats after remobilization using therapeutic ultrasound.

MATERIALS AND METHODS

We used 21 male Wistar rats, aged 10 weeks, obtained from the Central Bioterium of the State University of Western Paraná. They were kept in polypropylene boxes, with access to water and food at will, room temperature maintained at 22 °C and a 12-hour light/dark photoperiod, grouped into three animals per box. The study was conducted in accordance with International Standards and approved by Unioeste's Animal Use Ethics Committee (approval number - 09-21 – date of approval December 07, 2021).

The animals were randomly separated into 3 experimental groups with 7 animals in each group:

- Immobilization group (IG): they were immobilized and euthanized after the 3rd week of immobilization.
- Immobilization and Free Remobilization Group (FRG): they underwent the immobilization protocol and remained for 3 weeks without receiving any type of treatment.
- Immobilization and remobilization with ultrasound group (USG): they underwent the immobilization protocol and received ultrasound treatment for 3 weeks during the remobilization period.

Immobilization protocol

To perform the immobilization, the animals were anesthetized and immobilized with a plaster bandage according to a model adapted by Wutzke *et al.* (4). The tibialis anterior muscle was immobilized in an elongated position for 3 weeks. The orthosis was molded from the abdominal region, just below the last ribs, and then to the right pelvic limb of each animal, with the knee joint in 120° flexion and the ankle in total plantiflexion.

Therapeutic Ultrasound Protocol

The Sonoplus Ibramed® device, which was certified for calibration during the research period, was used to carry out the ultrasound therapy. The parameters used were: effective radiation area (ERA) of 1 cm², continuous emission, frequency of 1.0 MHz, power density of 0.5 W/cm², for 2 minutes on the knee (1 min on the lateral side and 1 min on the medial side) and 2 minutes on the ankle (1 min on the lateral side and 1 min on the medial side) of the right pelvic limb of the animals belonging to G3, three times a week for three weeks, totaling 9 sessions.

Euthanasia of the animals, preparation of slides and histopathological analysis

The animals in group G1 were euthanized after 3 weeks of immobilization, while the other groups (G2 and G3) were euthanized after 3 weeks of remobilization. For euthanasia, the animals were previously anesthetized with an intraperitoneal injection of ketamine hydrochloride and xylazine and, after checking their state of consciousness by clamping the interdigital folds, the animals were decapitated using a guillotine. The tibialis anterior muscle of the right pelvic limb was collected and fixed in Metacarn (70% methanol + 20% chloroform + 10% glacial acetic acid) for 2 hours and stored in 70% alcohol, then processed for inclusion in histological paraffin. The muscles were cut transversely at a thickness of 7 μm using an Olympus CUT 4055 microtome, and the slides were stained in hematoxylin and eosin to measure the histopathological parameters.

Histopathological index

The histopathological index classifies lesions into three categories: severe, moderate or mild. The formula used to calculate the histopathological index was $X = a \times w$. In this formula, "X" represents the total sum of the damage observed in the muscle tissue. The "a" parameter corresponds to the score that measures the extent of the lesion, where 0 indicates no lesion, 2 represents minimal lesion, 4 indicates moderate lesion and 6 represents severe lesion. The "w" parameter refers to the pathological importance

factor attributed to each lesion, which can be minimal, moderate or major.
The score obtained using the formula determines the injury index, and the higher the score, the greater the extent of the tissue damage. The injury index can vary, reaching a maximum of 320 points, depending on the characteristics and severity of the injuries present in the muscle tissue analyzed. The slides were analyzed using light microscopy and the changes observed were recorded in a Microsoft Excel® table. The structures found were measured and classified according to the index into: inflammatory and circulatory disorders - hemorrhage, edema, exudate and inflammatory infiltrate; regressive changes - rounded, angulated, divided fibers, with degeneration, vacuolization, atrophy, necrosis, myonuclei, increased nuclei, adipose tissue, altered nerve tissue and muscle spindles; and progressive changes - hypertrophy or hyperplasia of muscle tissue, hypertrophy or hyperplasia of connective tissue and neoplasms (**figure 1**).

Statistical analysis

Statistical analysis was carried out using the SPSS 20.0® program. Comparisons were made using Generalized Linear Models, with Fisher's post-test (LSD) applied. The significance value adopted was $p < 0.05$ and the results were presented as mean and standard deviation.

RESULTS

When analyzing the histopathological index, significant differences were observed between all the groups. IG had the highest score compared to the others, with an average of 106 points. On the other hand, FRG had an average score 65 points higher than USG, which had an average of 20 points. These results indicate important variations in the lesions and alterations observed in the muscle tissues between the groups analyzed.
A number of tissue alterations were observed in the IG, with the following standing out: circulatory disorders, a centralized nucleus, a basophilic halo, connective tissue hypertrophy and tissue disorganization in both connective and muscle tissue. These alterations indicate a significant compromise in the structure and integrity of the tissues analyzed in the group.
When comparing the groups, important differences were observed in the alterations. Group FRG showed less tissue disorganization and a basophilic halo compared to group IG, suggesting a reduction in the inflammatory process in this group. On the other hand, group USG showed a decrease in connective tissue hypertrophy and a major circulatory disorder, although without the presence of edema. In addition, a significant decrease in the number of cells undergoing tissue necrosis was identified in the USG. These results indicate that USG showed an improvement in the inflammatory response compared to groups IG and FRG. These findings highlight the importance of considering the different responses and developments observed in the different groups during histopathological analysis.

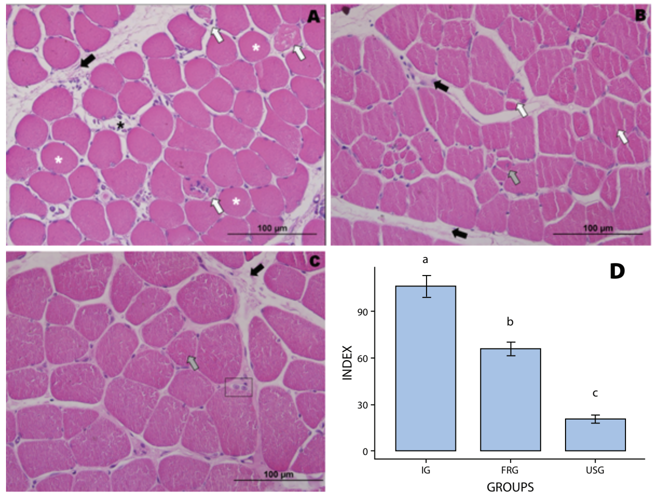

Figure 1. (A) Immobilized group (IG) the black arrow representing hypertrophy and disorganization of connective tissue, the white arrow showing cell necrosis, the black asterisk indicating the presence of inflammatory cells, the white asterisk representing rounded fibers; **(B)** Free remobilization group (FRG) the black arrow representing hypertrophy and disorganization of connective tissue, the white arrow showing cell necrosis, the grey arrow showing muscle atrophy; **(C)** The ultrasound remobilization group (USG), with the black rectangle showing satellite cells in division, representing muscle remodeling, and the black arrow representing hypertrophy and disorganization of the connective tissue; **(D)** Represents the standard deviation shown in the histopathological index, representing the degree of injury in each group.

DISCUSSION

This study evaluated the histopathological changes in the tibialis anterior muscle of Wistar rats after remobilization using therapeutic ultrasound for 3 weeks, in order to confirm its effectiveness. The histopathological index presented by Zazula *et al.* (22), was used for this purpose. This approach allows a detailed assessment of changes and lesions in muscle tissue, providing relevant information for understanding the damage that occurred in the context of this study, and an index with a significantly lower value was observed in the ultrasound-treated group, indicating a better restoration of tissue patterns within normality, *i.e.* less indicative of tissue damage.

The use of ultrasound is important in the musculoskeletal system, both diagnostically (23) and therapeutically (18). In this experiment, continuous therapeutic ultrasound was used, as this can provide a thermal effect, acting directly on tissue extensibility, reducing contractures and increasing local circulation (18, 24-27). In addition to these, ultrasound is also an important anabolic stimulus, acting on changes in cellular ion flow (mainly related to calcium, which in this case has anabolic rather than catabolic effects, which is also one of its functions in skeletal muscle) (28-33), resulting in, among other effects, a reduction in interleukin activity, an increase in the concentration of growth factors and protein synthesis, neovascularization and analgesia (27, 34-40).

All the effects mentioned above are extremely important during the remobilization period, as immobilization leads to a decrease in protein synthesis and an increase in protein degradation, with consequent muscle atrophy (1, 41), meaning that the anabolic stimulus of ultrasound is favorable to the trophic recovery of muscle tissue. Furthermore, since immobilization generates pro-inflammatory tissue characteristics, a reduction in angiogenic stimulus, as well as the replacement of contractile tissue by fibrous and fatty tissue (2, 3, 41), ultrasound may have an important action against these characteristics (37, 42). Also to be taken into account is the nociceptive alteration that immobilization generates (4), which in turn can affect the dorsal root reflex and the axonal reflex, promoters of neurogenic inflammation (43, 44), or be precisely caused by them. Regardless of the initial cause, the analgesic effects that ultrasound promotes (37) can prevent this configuration and thus produce effects such as those observed in the study of reducing the pathological tissue level, which was observed for GI and GRL that showed muscle fiber necrosis, hypertrophy and connective tissue disorganization. These findings show the effect of therapeutic ultrasound, which reduced connective tissue hypertrophy, muscle remodeling and the number of cells undergoing necrosis.

The absence of biochemical analysis which could indicate changes in interleukins and other inflammatory factors (45) is a limitation of the study, and suggestions for future studies which will be crucial to deepen the understanding of therapeutic ultrasound, including dosimetric curves, and its application in muscle rehabilitation, thus contributing to the development of more effective rehabilitation strategies to promote the recovery and adaptation of muscle tissue in situations of injury or functional incapacity. These suggestions are also aimed at advancing research towards clinical practice in humans. However, it is worth highlighting the novelty of this study, in which for the first time the histopathological index was used to analyze the muscle remobilization process.

CONCLUSIONS

It is concluded that therapeutic ultrasound has the potential to reduce the cellular inflammatory process, as evidenced by the reduction in connective tissue hypertrophy and the number of cells in the necrotic process in the tibialis anterior muscle.

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DATA AVAILABILITY

Data are available under reasonable request to the corresponding author.

CONTRIBUTIONS

AJKF, CSO, GPO, JMN, RSR, LFCR, DPA, GRFB: conceptualization. AJKF, CSO, GPO, JMN, RSR: data collection. LFCR, DPA, GRFB: results analysis and interpretation. AJKF, CSO, GPO, JMN, RSR: writing – original draft. LFCR, DPA, GRFB: writing – review & editing.

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