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Morphological effects of electrical stimulation and intermittent muscle stretch after immobilization in soleus muscle

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Summary. The objective of the present study was to assess the effectiveness of a combined protocol of muscle stretching and strengthening after immobilization of the hindlimb. Thirty female Wistar rats were divided into 6 groups: group immobilized for 14 days to cause full plantar flexion by cast (GI, n=6); group immobilized/stretched (GIS, n=6): submitted to the same immobilization and to 10 days of passive stretching; group immobilized/electrically stimulated (GIES, n=6): similarly immobilized and submitted to 10 days of low frequency electrical stimulation (ES); group immobilized/stretched/electrically stimulated (GISES, n=6): similarly immobilized, submitted to 10 days of stretching and ES application; group immobilized/free (GIF, n=3): similarly immobilized and then left with free limbs for 10 days; control group (CG, n=3). The middle portion of the soleus muscle was frozen and sections were stained with HE or mATPase. Morphological analysis revealed high cellular reactivity in the GISES, GIES and GIS groups. The lesser diameter and proportion of type I fibers (TIF) and type II fibers (TIIF) (at pH 9.4) and connective area (at HE stain) were measured with an image analyzer and the data obtained were analyzed statistically by the unpaired Student t-test ($p \leq 0.05$). The results indicated that: a) immobilization generated atrophy of both fiber types ($p<0.05$); b) joint application of ES and stretching was not efficient in reestablishing the size of the two fiber types compared to CG ($p<0.05$); c) the ES protocol reestablished only the size of TIIF, which showed values similar to those detected in CG ($p<0.05$); d) the stretch increased the proliferation of the perimysium connective tissue ($p>0,05$). Thus, we conclude that, in the model applied here to female rats, a stretching protocol may limit the volume protein gain of soleus muscle fibers and increase the connective interstitial tissue.

Key words: Neuromuscular electrical stimulation, Stretching, Immobilization, Soleus, Rat, Morphology

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Introduction

The skeletal muscles of mammals may suffer adaptations of a reversible or irreversible nature on the basis of the positions to which they are exposed daily. The conditions most frequently observed on the longitudinal axis are shortening (retractions, of a dynamic character) and contractures (of a static character). Atrophy and hypertrophy/hyperplasia are observed on the transverse axis. Abnormalities on both axes are frequently observed in sedentary individuals or after situations of immobilization of a body segment which impairs the functional excursion of the muscles involved, and of the joint and the development of strength (Gajdosik, 2001).

It has been reported that muscle atrophy occurs 48 hours after the beginning of immobilization (William and Goldspink, 1971; Gamrin et al., 1998), with a 37% loss of muscle mass being observed after 7 days (Zemková et al., 1990; Herbert and Balnave, 1993; Kannus et al., 1998). A reduction in total protein synthesis was observed, as well as a reduction of mRNA content of specific enzymes involved in protein metabolism such as carbonic anhydrase III phosphoglucoisomerase, and of alpha-actin (Howard et al., 1989; Thomason et al., 1989; Brownson and Loughna, 1996). Chronic immobilization of animals for 4 to 6 weeks has demonstrated the transformation of slow (oxidative) soleus fibers to fast oxidative-glycolytic fibers (Fitts et al., 1989).

Muscle immobilization in a shortened position generates a decrease in the total force produced, resulting from decreases in both the active and the

passive resistive forces (Alder et al., 1959; Williams and Goldspink, 1978), favoring the rupture of cellular or subcellular structures upon application of muscle tension or stretching (Jarvinen et al., 1992).

Longitudinal fiber reduction and a consequent decrease in series of total sarcomere numbers have also been reported (Williams et al., 1988; Williams, 1990; Goldspink, 2002), with accumulation of perimysial connective tissue after 2 days of immobilization followed by accumulation of endomysial connective tissue after 7 days of immobilization (Williams and Goldspink, 1984). Other studies by the same authors have reported that muscle stretching (muscle tension in extension) can cause a longitudinal increase of the muscle by the addition of sarcomeres in series and by the reduction of connective tissue proliferation along the muscle fibers (Godspink et al., 1968, 1971). The same group also demonstrated that the addition of sarcomeres is greater when stretching is applied daily and maintained for a period of 30 minutes (Williams et al., 1988). However, proliferation of connective tissue is limited when the muscle is stretched at 2 day intervals during a period of immobilization of 10 days (Williams et al., 1988). Other studies have demonstrated that stretching favors the longitudinal growth of muscle fibers and reduces the proliferation of connective tissue after 15 and 21 days of immobilization of the lower limbs of rats in a shortened position, and also partially prevents the atrophy of muscle fibers (Gomes et al., 2004). In another view, mechanical properties such as stretching to the elastic limit, stiffness and elastic energy of the gastrocnemius muscle returned after a stretching protocol applied during a period of 10 days to rats previously immobilized for 2 weeks (Carvalho, 2004).

Neuromuscular electrical stimulation (ES) is a method commonly used to minimize the damage caused by immobilization. During the application of this technique, type II fibers (TIIF) are first recruited in muscles of different compositions and functions (Delitto and Snyder-Mackler, 1990; Sinacore et al., 1990; Robinson and Snyder-Mackler, 1995), permitting a return to the ability to generate force by the stimulated muscle (Binder-Macleod et al, 1995).

When this technique is applied during the period of immobilization, no increase in connective tissue is observed in the muscle, but the loss of sarcomeres is not prevented (Williams et al., 1988). The isometric and isotonic contractile properties of different muscles with a predominance of fast and slow fibers were evaluated after 6 weeks of immobilization (Witzmann et al., 1982). After submitting the muscles to a protocol of electric stimulation, the authors observed that muscles with a predominance of slow-twitch fibers (type I) require about 4 days to recover their isometric contractile properties, whereas muscles composed of fast fibers (types IIa and IIb) recover after 14 days. However, Lucas et al. (1992) reported that the latissimus dorsi muscle of dogs submitted to ES for 4 weeks, 24 h/day, 30 Hz, presented an increase in the number of type I fibers (TIF) and a progressive decline of TIIF, although the diameter of the latter did not change. When stimulation was maintained for 12 weeks there was an increase in mitochondrial volume in each muscle fiber. Thus, the application of ES in an uninterrupted or intermittent manner results in a differential recruitment of muscle fibers. Carvalho (2004), using ES (frequency of 50 Hz) in gastrocnemius muscle shortened and immobilized for 14 days, observed a return of the mechanical properties (load and stretching in the limit of proportionality and in the maximum limit, ultimate strain, stiffness and elastic energy similar) when compared with a control group.

The objective of the present study was to assess the effectiveness of some combined physiotherapeutic protocols similar to those used in clinical practice for humans after immobilization of a segment for 14 days. To this end, the trophic conditions of the fibers and the proportion of muscle fiber types and connective tissue area were assessed after the joint use of stretching and low-frequency neuromuscular ES, and after the simple application of periodic sessions of passive stretching.

Material and methods

Twenty-seven female Wistar rats (weight, 214.±36.5 g) were submitted to immobilization of the left hind limb with a plaster cast with shortening of the sural triceps muscle according to the protocol described by Booth and Kelso (1973), with some adaptations. The animals were anesthetized intraperitoneums with 60 mg/kg ketamine hydrochloride and 15 mg/kg xylazine. The limbs were wrapped with a tubular mesh and cotton bandages in order to avoid ulcerations and then encased in a 2.5 cm wide quick-drying plaster cast applied in a standard manner. The animals were divided into the following groups: immobilized group (GI, n=6) with the hind limb left in a full flexed position for 14 days; immobilized/stretched group (GIS, n=6) similarly immobilized for 14 days and then allowed to move freely in their restricted cages and submitted to passive manual stretching for 10 days (10 repetitions with 15 seconds of stretching); immobilized/electrostimulated group (GIES, n=6), similarly immobilized for 14 days and then allowed to move freely in their restricted cages and submitted to low-frequency ES of the hind limb for 10 days; immobilized/ES/stretched group (GISES, n=6), similarly immobilized for 14 days and then allowed to move freely in their restricted cages and submitted to ES application and passive manual stretching of the hind limb (10 repetitions with 15 seconds of stretching) for 10 days; immobilized/free group (GIF, n=3) submitted to similar immobilization for 14 days and then allowed to move freely in their cages for 10 days, and control group (CG, n=3), consisting of animals submitted to no intervention. The animals were sacrificed on the day after the end of training. The soleus muscle was immediately isolated and a fragment of the central portion was obtained and frozen in liquid nitrogen. Five-

µm thick sections were obtained, stained with Hematoxylin-Eosin (HE) or processed for myosin-ATPase (mATPase) at acid and basic pH. Morphologic analysis was performed with a standard light microscope and morphometry was performed with an Image-Pro Plus analyzer (Olympus, version 4.1 for Windows, Media Cybernetics), considering the lesser diameter of 50 TIF and 50 TIIF. The proportion of TIF and TIIF was determined by analyzing 5 random fields on the mATPase slides per animal. Connective tissue area was measured using 3 random fields on the HE slides per animal. Data were analyzed statistically by the unpaired Student t-test, with the level of significance set at 5% ($p \leq 0.05$).

Neuromuscular electrical stimulation

An IBRAMED current generator, model NEURODYN II Compact®, was used for therapeutic intervention by means of neuromuscular ES. The characteristics of the instrument are as follows: pulse frequency range (R) from 10 to 166 Hz; on cycle of 2 to 20 seconds (s); off cycle of 3.5 to 38 s; time of pulse train increase: 2.5 s; time of pulse train decrease: 0.5 s; amplitude range: 0 to 80 mA; pulse duration varying automatically from 25 to 280 µs, and an asymmetrical biphasic square wave.

The following parameters were used to stimulate the animals: frequency of 50 Hz, on cycle 13 s, off cycle of 22 s, and an intensity of approximately 1 mA (resistance of 100 Ω) able to promote a sustained and visible contraction of the muscle. This frequency range is used in order to re-establish the ability to generate force by the electrostimulated muscle because it preferentially recruits type II fibers (Enoka, 1988; Delitto and Snyder-Mackler, 1990; Sinacore et al., 1990; Heyters et al., 1994). Before application of ES, the animals were shaved in the dorsal-inferior region and in the ventral portion of the right gastrocnemius muscle and anesthetized with 60 mg/kg ketamine hydrochloride and 15 mg/kg xylazine by the intramuscular route. ES was performed after the immobilization period, in the morning, for 10 consecutive days for 10 minutes per day. After the application of the protocol(s), the animals remained with restricted movement in the cages until the next day.

The current was transmitted to the animals through two cables and two electrodes (carbon-silicone). A fine layer approximately 1 mm thick of hydrophilic gel at neutral pH was first applied to a rectangular 5x3 cm electrode fixed in the dorsal-inferior region of the animal. Another electrode used for local stimulation of the gastrocnemius muscle was a pen developed on a smaller scale, with a gel layer being also used for adequate conduction of current to the muscle.

Stretching technique

The manual passive stretching technique was performed by applying manual force to the plantar region in the dorsal flexion direction, stretching the triceps muscle of the rat's right hind limb for 15 seconds, measured with a chronometer. The technique consisted of a single series of 10 passive exercises separated by 30 seconds of rest over 10 consecutive days.

The same criteria used for ES and passive manual stretching were used for the GISES group, but with ES being performed first and stretching later.

Results

Morphology

Morphological analysis of CG demonstrated a predominance of TIF and a discrete irregularity in fiber size (Fig. 1A), with TIIF being smaller than TIF. For GI, using HE staining, the fibers revealed variable irregularity of fiber types with some splitting (50% of the animals), with occasional findings of hyaline necrosis and regenerating fibers (16% of the animals) (Fig. 1B). The soleus muscle of the animals in the remaining groups exhibited reactive changes ranging from discrete to intense. The most reactive group was GISES, with a larger number of rats presenting changes such as nuclear centralization, splitting and marked irregularity of fiber size (67% of the animals), TIIF of reduced size, myophagocytosis and perimysial increase of connective tissue (50% of the animals), hyaline necrosis, basophilic halos, and regenerating fibers (33% of the animals) (Fig. 1C). The GIES group presented mild to moderate changes including TIIF of varying size (83% of the animals), regeneration, nuclear centralization and a predominance of TIF (50% of the animals), hyaline necrosis, a basophilic halo, and lobulated fibers (33% of the animals) (Fig. 1D). The GIS group presented occasional splitting and TIIF of reduced size (83% of the animals); regenerating fibers and connective tissue increased in the perimysium (66% of the animals), and a mild increase of the other discrete and minor findings such as a basophilic halo and nuclear centralization (Fig. 1E). The GIF group showed irregularity of fiber size, mainly for TIF (Fig. 1F). The enzymologic method of mATPase identification at pH 9.4 showed a variable number of fibers with intermediate staining, classified as hybrid.

Morphometry

The mean lesser diameter values obtained for each group are illustrated in Fig. 2A,B. Statistical analysis for the comparison of mean lesser diameter values among groups indicated a statistically significant difference in TIF between CG X (GI; GIS; GIES; GISES, $p< 0.01$), GIS X GIF ($p<0.05$) and between GI X GIF ($p<0.05$) (Table 1). Significant differences in TIIF were observed between groups CG X (GI; GIS; GISES, $p<0.01$), GI X (GIES; GIF, $p<0.05$), GIS X GIES ($p<0.05$), and GIES X GISES ($p<0.01$) (Table 1).

The results concerning the lesser diameter of hybrid fibers (HF) could be compared only between groups CG, GI, GIS, GIES and GISES. There was a significant difference between GI X (GIES, GISES, $p<0.05$), GIS X (GIES, GISES, $p<0.05$) (Table 1).

The results regarding the proportion of fibers are illustrated in Fig. 3. Analysis of this figure indicates a tendency to a reduction of TIF numbers in groups GI,

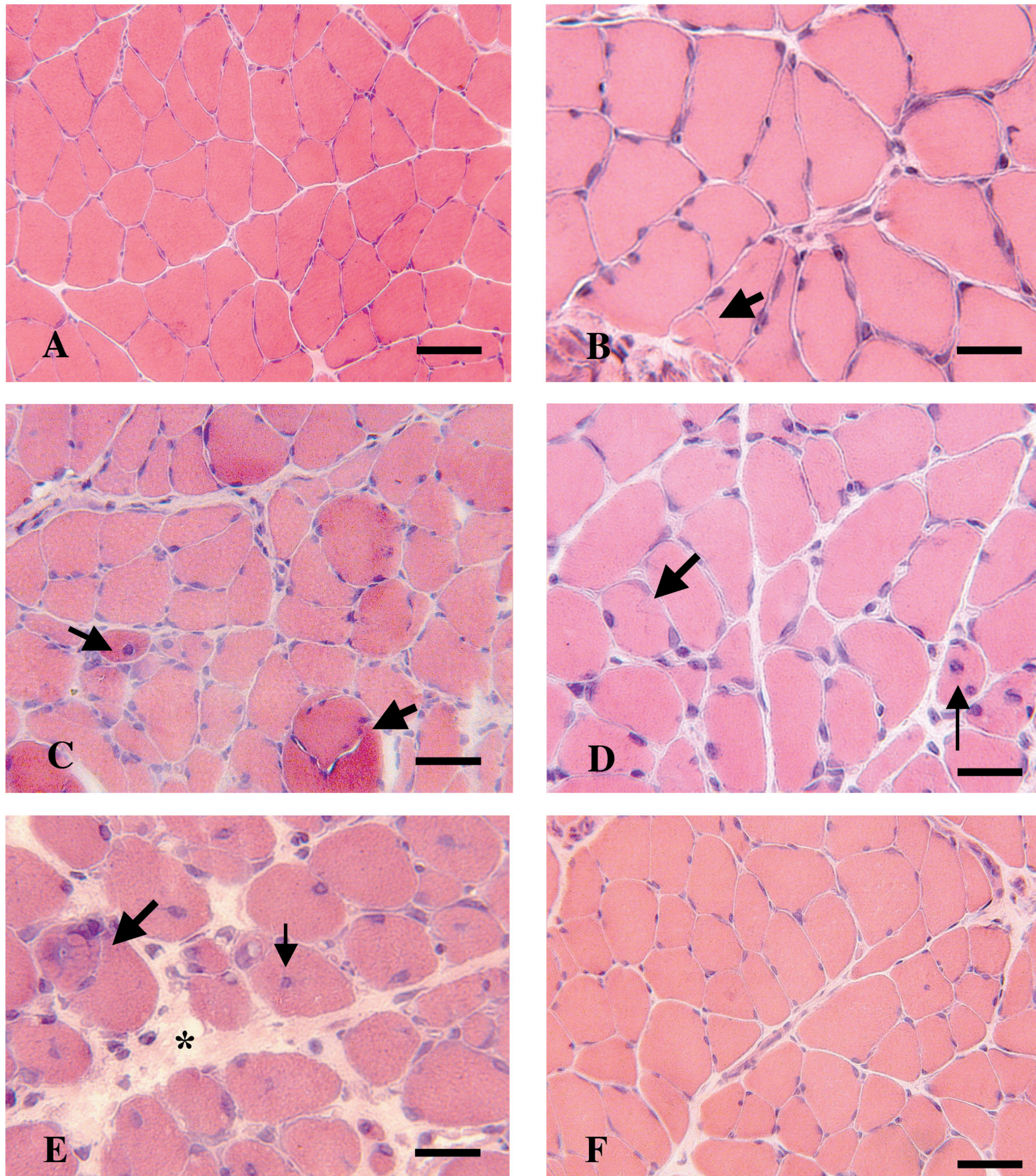
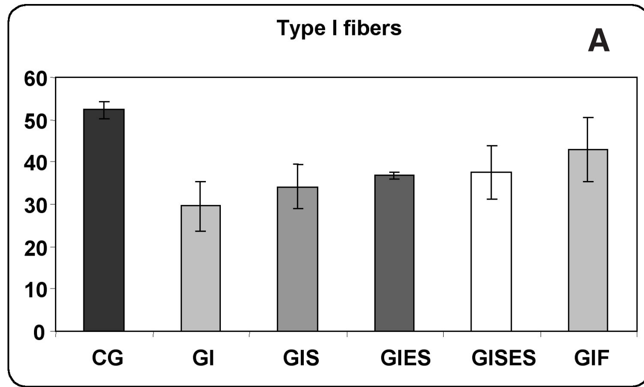


Fig. 1. Transverse sections of the soleus muscle stained with HE reaction. **A.** Micrograph of CG showing fibers of uniform size and with peripheral nuclei (HE) (Bar: 80 µm). **B.** Micrograph of GI presenting fibers with irregular shape and cell splitting (arrow) (Bar: 25 µm). **C.** Micrograph of GIES presenting irregularity of fiber size, centralized nuclei (arrow) and cell splitting (large arrow) (Bar: 60 µm). **D.** Micrograph of GISES showing some irregularity of fiber size, centralized nuclei (arrow), discrete basophilic halo (large arrow) (Bar: 40 µm). **E.** Micrograph of GIS showing centralized nuclei (arrow), cell splitting (large arrow) and increase in the connective tissue (*) (Bar: 40 µm). **F.** Micrograph of GIF presenting discrete irregularity of fiber size (Bar: 50 µm).



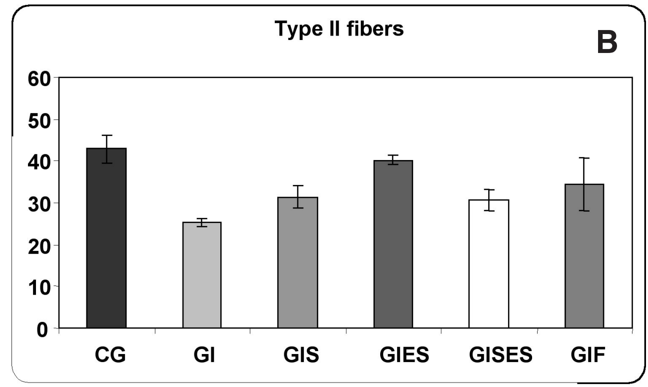


Fig. 2.A. Mean lesser diameter of type I fibers in the different groups studied. **B.** Mean lesser diameter of type II fibers in the different groups studied.

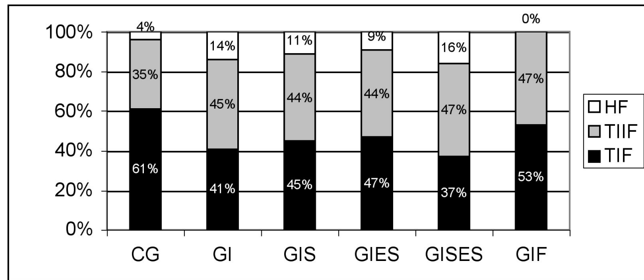


Fig. 3. Percentage of TIF, TIIF and FH in the different groups analyzed.

Table 1. Mean lesser fiber diameter in the different groups studied.

	TIF	TIIF	HF
CG	52.27 ± 2.0	42.79 ± 3.2	16.7 ± 6.82
GI	29.60 ± 5.9 *	25.33 ± 0.9 *	12.1 ± 7.76
GIS	34.25 ± 5.1 *	31.38 ± 2.8 *	15.7 ± 9.94
GIES	36.86 ± 0.7 *	40.26 ± 1.1 # ° ◊	35.45 ± 8.9 # °
GISES	37.48 ± 6.3 *	30.57 ± 2.5 *	28.57 ± 5.1 # °
GIF	42.85 ± 7.6 #	34.44 ± 6.3 #	0 ± 0

*: p<0.05 (compared with control); #: p<0.05 (compared with GI); °: p<0.05 (compared with GIS); ◊: p<0.05 (compared with GISES).

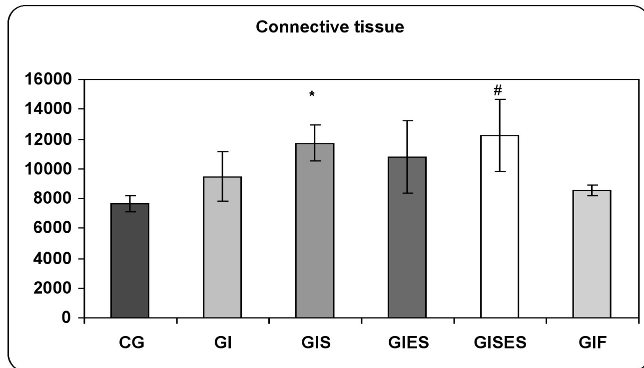


Fig. 4. Perimysial connective tissue mean area in the different groups studied. *: p<0,05 (Compared with CG, GI, GIF); #: p<0,05 (Compared with CG, GI, GIF).

GIS, GIES, GISES (not significant-NS) and a slight increase in TIIF and HF (NS).

Statistical analysis of the connective tissue area showed significant difference between GIS x (CG, GI, GIF, p<0.05) and GISES x (CG, GI, GIF, p<0.05) (Fig. 4). These results suggest that the stretch employed every day induced connective tissue proliferation in the perimysium.

Discussion

This is the first morphological study to quantify muscular and interstitial effects under combined protocols of rehabilitation after immobilization conditions. The results of the present study demonstrated that immobilization generates TIF and TIIF atrophy and that among the protocols used, only the type II fiber size of GIES was not significantly different from CG. This was probably due to the fact that these fibers, regardless of the frequency used in ES, are initially recruited in muscles of different compositions (Enoka, 1988; Delitto and Snyder-Mackler, 1990; Sinacore et al., 1990; Heyters et al., 1994). The data obtained with the protocols that used the stretching technique, including those combined with ES, showed significant variation in TIIF size compared with the CG despite the high cell reactivity observed. The present study also demonstrated that the release in the cage after the period of immobilization of the animals helps reduce the cellular changes since: (1) the muscles of this group (GIF) presented low cellular reactivity in the morphological

analysis and (2) statistically significant difference in the lesser diameter of TIF and TIIF was observed between GI and GIF but not between CG and GIF.

Okita et al. (2001) reported that passive stretching maintained for 30 minutes and performed 6 times a week for 3 weeks promoted an increase in TIF diameter compared to an immobilized group and to a group immobilized and released during the first week of treatment. On the other hand, these investigators did not observe a significant difference between the data for the group submitted to passive stretching and those for the control. The result of weekly application of passive stretching for 40 minutes in the presence or absence of immobilization was evaluated 21 days after the beginning of the protocol, and atrophy of both oxidative and glycolytic fibers was observed (Gomes et al., 2004). A parallel study using ultrastructural analysis of the muscles of these same animals and on the muscles of animals submitted to passive stretching at 3 day intervals showed that weekly stretching generated less damage to the myofilaments and less rupture of the Z lines compared to the muscles of the animals submitted to stretching at 3 day intervals (manuscript submitted). In the present study we did not observe any signs of muscle hypertrophy caused by the application of the stretching technique. On the other hand, we observed intense cellular reactivity in the muscles of animals submitted to stretching, a fact representing degenerative and/or regenerative states triggered by the passive stretching movement executed by the investigator. There are numerous studies demonstrating that the neuromuscular system can adapt to environment situations, especially after atrophy (Edgerton et al., 2002). As a consequence, morphological reactions such as cell damage are observed. The above results suggest that the time and duration of application of the stretching technique may be important factors influencing the responses of the muscle fibers in terms of protein synthesis or degradation.

In the present study, the mean lesser TIIF diameter of the control group was wider than that observed for the experimental groups, except for GIES (not significant - NS). These results indicated that ES may cause hypertrophy of this fiber type within a short period of time, probably due to the fact that neuromuscular ES preferentially recruits TIIF, as reported by Kramer (1987). When followed by stretching, the ES protocol (GISES) was found to be less effective than the protocol of ES alone (GIES), since the lesser diameters of the two fiber types of GISES were not statistically similar to those of CG. Using a biomechanical approach, Carvalho (2004) observed that ES used after immobilization of the limb for 14 days was effective in re-establishing all the properties of control group, but observed partial results for the stretched group. Goldspink (2002) demonstrated an additive effect of these protocols in anterior tibial muscle in terms of promoting rapid fiber hypertrophy. Perhaps the technical procedures adopted in the cited report, as well as the different phenotypic characteristics of the muscles studied, may have favored the results obtained by those investigators.

Statistical analysis of the mean lesser HF diameter of the different experimental groups indicated a significant increase in GIES and GISES compared to GI and GIS. Again, ES must have been the factor that induced a modification in the lesser diameter of these fibers, as was also the case for TIIF. Although the procedures applied in the present study do not permit us to infer about the presence of different types of myosin heavy chains (MHC) in this cell type, we may raise the hypothesis that the cells contained a larger quantity of type II MHC, since they were more responsive to ES.

The experimental groups submitted to immobilization tended to show a reduction in the proportion of TIF compared to CG. These results agree with those reported by Booth and Kelso in 1973. These authors assessed the contractile and histochemical properties of the femoral rectus and soleus muscles of rats after 4 weeks of immobilization and observed a reduction in the amount of TIF and an increase in intermediate fibers (IIa). Immobilization in a shortened position, as well as tenotomy, can cause fiber transition from TIF to TIIF (Unsworth et al., 1982; Fitts et al., 1989). Studies conducted by Loughna et al. (1990) have demonstrated up-regulation of the MHCIIb gene in 5-day shortened-immobilized soleus muscle. Allen et al (1996) observed an increase in the percentage of the fibers expressing a fast phenotype based on their MHC in single fibers of the soleus muscle after spaceflight. Our results also demonstrated an increase in the quantity of fibers with intermediate staining at pH 9.4, a fact possibly indicating states of fiber transition. Fiber transition seems to depend on the muscle stimulated, on the animal species, and on the method applied.

In addition, it has been observed that the duration of chronic low-frequency stimulation is an important factor in the transformation of TIIFb to TIF (Pette and Staron, 1997) and that the inverse transformation is only possible by denervation followed by high-frequency phase stimulation because the muscle receives its slow-type neural input (Lömo et al., 1980). In contrast, stretching and a mechanical overload do not seem to cause an increase in neuromuscular activity (Pette and Staron, 1997); thus, transformation from TIIFb to TIF has been reported, being more visible when the muscle presents a high proportion of glycolytic fibers (Loughna et al., 1990; Goldspink et al., 1992). In the present study, we observed a tendency to an increase in TIIF proportion and a tendency to a reduction in TIF proportion in the immobilized groups compared to control (NS), and an increase in the proportion of fibers with intermediate staining in the groups submitted to immobilization (NS). On the other hand, no significant changes in the proportions of TIIF and TIF were observed in the muscles of rats submitted to ES application and/or stretching compared to the group that was only immobilized. Perhaps the time and the duration of the application of these methods (ES and/or

stretching) were not sufficient to determine alterations in the proportion of fibers described in the literature, or the muscle under study (soleus), by having a predominance of TIF, did not preserve a significant phenotypic variation, as indicated in the literature.

Other information produced in this study was about the connective tissue under immobilization and the influence of stretching and/or ES after immobilization. First, the muscle atrophy appears to be correlated with an altered turn-over of the connective tissue extracellular matrix components in muscle inactivity, such as suspension of the rats' hindlimbs (Giannelly et al., 2005). Studies conducted by Goldspink and collaborators have shown that stretching techniques used together with immobilization could prevent connective tissue proliferation. Our quantitative results demonstrated that 14 days of immobilization were not sufficient to induce significant connective tissue proliferation but the stretching used after the limb restriction was. Despite the apparent negative effects of stretching, Carvalho (2004) pointed out that the biomechanical assay improved the mechanical responses of the stretched muscles compared to the muscles only immobilized.

We may conclude that, after 14 days of soleus muscle shortened immobilization, (1) 10 days of neuromuscular ES at a frequency of 50 Hz could reverse the size of TIIF, but not of TIF; (2) 10 days of stretching, employed exclusively or combined with ES, did not reverse the size of the two fiber types to control values; (3) stretching employed every day induces connective tissue proliferation in the perimysium. Other techniques will be explored as well as other combined protocols to confirm that stretching and ES would be employed at different times during the therapy.

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