



Twice a day neuromuscular electrical stimulation post-immobilization alters the mechanical properties and the histomorphometry of the skeletal muscle

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Abstract

Background: The responses to the use of neuromuscular electrical stimulation (NMES) technique, once or twice a day, are not well described in literature. In this direction, we measured the biomechanical properties and morphological responses of NMES, applied once or twice a day to the gastrocnemius muscle of female rats after cast immobilization in comparison with both sham control and immobilized control groups.

Methods: Eighty Female Wistar rats (Body mass \pm SDg; 210 ± 10 g) were divided into five groups: Control (CG); Immobilized (IG); Immobilized then freed (IFG); Immobilized and subjected to NMES once a day (1ESIG) and another twice a day (2ESIG). All groups that suffered cast immobilization were kept in a shortened position for fourteen days, and then NMES was applied once or twice a day for ten consecutive days. The assessments included the mechanical properties of load and elongation at the limit of proportionality, as well as the resilience obtained from the load versus elongation graph. The cross-sectional area (CSA), the different fiber type proportions and the perimysial connective area were measured.

Results: There was restoration of load at the limit of proportionality (LLP), but not resilience, in the 1ESIG and 2ESIG, as well as restoration of atrophy (CSA) of type I (TIF), type IIad (TIIadF) and type IIc (TIIcF) fibers in the 1ESIG, but not in the 2ESIG. An induction of the TIIcF proportions in the 1ESIG and 2ESIG, and a reduction of the TIIad in the 1ESIG were verified.

Conclusions: The muscle remobilization intensity can, influence the recovery responses post-immobilization either positively or negatively, for it was observed that NMES once a day can recover most mechanical (except resilience) and structural parameters of gastrocnemius muscle after casting, whereas twice daily, promoted deleterious effects to the much clearer muscles. Moreover, the animals' free movement allowed them to identify their own mobilization limits, which did not cause neither mechanical nor structural overload in the evaluated muscles.

Keywords: Immobilization, gastrocnemius muscle, electric stimulation, skeletal muscle

Introduction

Immobilization is known to promote deleterious changes in the skeletal muscle, such as cellular atrophy [1], reduction of extensibility, decrease in the amount of mitochondria, capillary density, and the ability to generate force as well as the increase in intramuscular connective tissues, among others [2]. The mechanical stress provoked by spontaneous or induced muscle

contraction regulates cellular activity by the mechanism of cell transduction, influencing the morphofunctional aspects [3,4] through a complex signaling cascade that involves membrane proteins, intermediate muscle filaments, and contractile proteins [5]. The cross-sectional fiber area and the myosin heavy chain (MHC) are components known to be influenced by variations in muscle tension. It has been reported that, short-

term training induces collagen expression possibly linked to a greater force production during concentric, eccentric and isometric contractions in association with these changes [6]. Consequently, these alterations modify the mechanical properties of the bone-tendon-muscle-tendon-bone complex [7,8], reinforcing or weakening the mechanical responses to contraction stress.

Techniques such as neuromuscular electrical stimulation (NMES) have been used before and after a given period of immobilization in an attempt to restore the ability of the muscles to support the tissues and to transmit external forces to them. The use of these techniques during immobilization triggers a considerable increase in glycogen reserves, maintains cellular tropism, reduces the proliferation of connective tissue [9] preserves the cross-sectional area [10] and protein synthesis [11] and re-establishes some mechanical properties of the striated skeletal muscle [12]. NMES is commonly applied at a once a day frequency during clinical rehabilitation practice [13,14], however currently, some studies show the possibility of using this resource for more than once a day in rehabilitation protocols [15-17]. Nevertheless, the specific tissue responses to this intensity of stimulation have not been described in the literature. Thus, the aim of the present study was to assess and compare the morphofunctional effects of NMES applied once or twice a day to the gastrocnemius muscle of female rats after 14 days of cast immobilization in a shortened position.

Methods

Groups

Eighty female Wistar rats (Body mass \pm SDg; 210 \pm 10g) were provided by the Central Animal Facilities of the University of São Paulo's (USP) Ribeirão Preto Campus. The animals were kept in pairs in plastic cages measuring 41x34x16 cm in a room with controlled temperature (24°C) on a 12 hours light/12 hours dark cycle, with free access to water and food in the Animal Facilities of the Bioengineering Laboratory at the Medicine college of the USP in Ribeirão Preto. The project was approved by the Ethics Committee for the Use of Animals (CEUA), Ribeirão Preto Campus, USP (Protocol n° 06.1.692.53.8) and followed the norms of the Guide for Care and Use of Laboratory Animals (National Research Council, 1996).

The animals were divided into groups of 16 rats each, with 10 animals being used for the mechanical assay and six for the histochemical analysis. The groups were set up as follow: the immobilized group (IG)–the right hind limb was immobilized for 14 consecutive days then the animals were euthanized; the immobilized and once a day electrostimulated group (1ESIG)–after immobilization for 14 days, the animals were subjected to NMES once a day for 10 days and then allowed to move freely in a cage; the immobilized and twice a day electro stimulated group (2ESIG)–similar to the previous group but electrostimulated twice a day; the immobilized and then free group (IFG)–the animals were immobilized for 14 days and then allowed to move freely in their cage for 10 days;

the control group (CG)–the animals were housed in a plastic cage for 14 days in order to match the period of contention of the animals in the remaining groups.

For the immobilization and NMES procedures the animals were anesthetized with a combination of ketamine hydrochloride (80 mg/kg) and xylazine hydrochloride (15 mg/kg), at the dose of 0.05 ml of the mixture per 100 g body mass through the intraperitoneal route. The application of this anesthetic mixture is often reported in different studies [10,11]. In this study, the present combination was adopted because of anesthesia time being a dependent of the dosage which minimized the effects induced by the animal's immobility in the referred period, knowing that the quantity of the applied anesthetic controlled and was enough only for the period of carrying out the procedures.

At the end of the experimental procedure the animals were weighed and sacrificed with an overdose of the combined anesthetics.

Immobilization procedure

The right hind limb of the animal was immobilized with a plaster cast applied to the pelvis, the hip (neutral position) and the fully extended knee, while the ankle joint was left in plantar flexion for 14 days. For details, see Mattiello-Sverzut et al. [10].

Procedure of NMES

A BIOSET (Bioset, São Paulo, Brazil) Physiotonus Four current generator with biphasic (depolarized) low frequency and pulses of short duration applied at controlled frequencies was used for NMES. The parameters used for stimulation were 50 Hz frequency, 8 s/22 s on/off cycles, 250 μ s pulse width, and an intensity of approximately 1 mA (measured with a resistance of 100 Ω , in a pilot study), capable of promoting a sustained and visible muscle contraction. Before the NMES procedure, the animals were shaved in the lumbar region and in the middle of the calf of the gastrocnemius muscle of the right hind paw in order to facilitate electric current conduction. Two electrodes were used for NMES, the first in a rectangular shape (area: 15 cm²) was fixed in the animal's lumbar region. The second electrode that was used, made of carbon-silicon in the shape of a pen (area: 0.29 cm²), was put on the middle of the calf.

After the 14 consecutive days of immobilization, the NMES protocol was applied for 10 minutes once a day (in the morning) or twice (in the morning and in the afternoon with a six hour interval) to the different groups.

Mechanical assay

The right hind limb of the animals subjected to the mechanical assay was dissected by hip disarticulation. The gastrocnemius muscle was exposed, preserving its origin in the distal third of the femur and its insertion in the heel. A special accessory was used for fixing and testing of traction, which allowed

the gastrocnemius muscle to undergo the trial preserving its muscular origin and insertion. For the trial a universal testing machine was used, brand EMIC[®]-DL10000 model (EMIC, São José dos Pinhais, PR, Brazil) equipped with a load cell of 10 Kgf. The adapted parameters for the feat were a pre-load of 0.30 Kgf where the rate of the test was 10mm/min and the load as well as the elongation limits were 8 Kgf and 25 mm respectively [12]. For each increase of load applied to the muscle a value was obtained resulting from elongation, which enabled the construction of graphs for the analysis of the mechanical properties of resilience, load and elongation in the proportionality limit. This limit is the last point in the linear segment of the load versus elongation graph. The coordinates obtained at this point represent the proportional load and elongation. Resilience is defined as the capability that the material has in absorbing energy in the elastic fase. In this study it was obtained through finding the area of the triangle beneath the load versus elongation curve during this fase.

Morphological and morphometric analysis

Two portions of the central region of the middle of the calf of the gastrocnemius muscle (medial and lateral) were frozen in liquid nitrogen and stored in a freezer at -80°C . For histological processing the fragments were cut into 5- μm thick cross-sections with a Leica CM 1850UV cryotome (Leica Microsystems Inc, Chicago, IL, EUA) at a temperature of -25°C . The sections were collected on 24x50 mm coverslips, stained with hematoxylin-eosin (HE) and processed for the determination of myofibrillar adenosine triphosphatase (mATPases, E.C.2.1.3.5.7.9.1) at pH 4.3, 4.6 and 9.9, in the Laboratory of Neuropathology of the Department of Pathology, FMRP-USP, according to routine methods for skeletal muscle processing [18,19]. The slides were analyzed qualitatively under a Leica DM 2500 light microscope.

Morphological analysis of muscle tissue was performed with HE. Percentage of morphological alterations (nuclear centralization, lobulated fibers, hyaline necrosis, cells on degeneration and regeneration, splitting cells, central core and basophilic halo) was determined considering the total number of cells for each fragment from each muscle in the groups studied.

The images of the slides processed at pH 4.6 were used for morphometric analysis. The images were captured with a Leica DM 2500 light microscope connected to a Leica DFC 300FX digital videocamera and to a microcomputer equipped with Qualiview-Atonus software (Atonus, São José dos Campos, SP, Brazil), which permitted cell count and measurement of the section area of the fibers. Three fields were evaluated in the deep region of each portion (lateral and medial) of the gastrocnemius muscle. The deep region of both portions was chosen for collection because it contains a higher concentration of different fiber types. According to Staron et al., [18], type I, Ic, IIc, IIad, IIld, IIldb and IIlb fibers are present in

the deep region of the gastrocnemius muscle. The quantity of perimysial connective tissue in the fields evaluated was determined using Qualiview. The margins and the areas of the cross-sections of the fibers and vessels were measured and subtracted from the total area of the field evaluated ($91860.51 \mu\text{m}^2$), and the result corresponded to the quantity of perimysial connective tissue.

Statistical analysis

Data are reported as means \pm SD. The Kolmogorov-Smirnov test was first applied to the results to assess the normality of the sample and was followed by ANOVA and by the Tukey-Kramer test when the sample was found to be normal. When non-normal distribution was observed, the data were analyzed by the Kruskal-Wallis test followed by the Dunnett test. The level of significance was set at 5% in all analyses.

Results

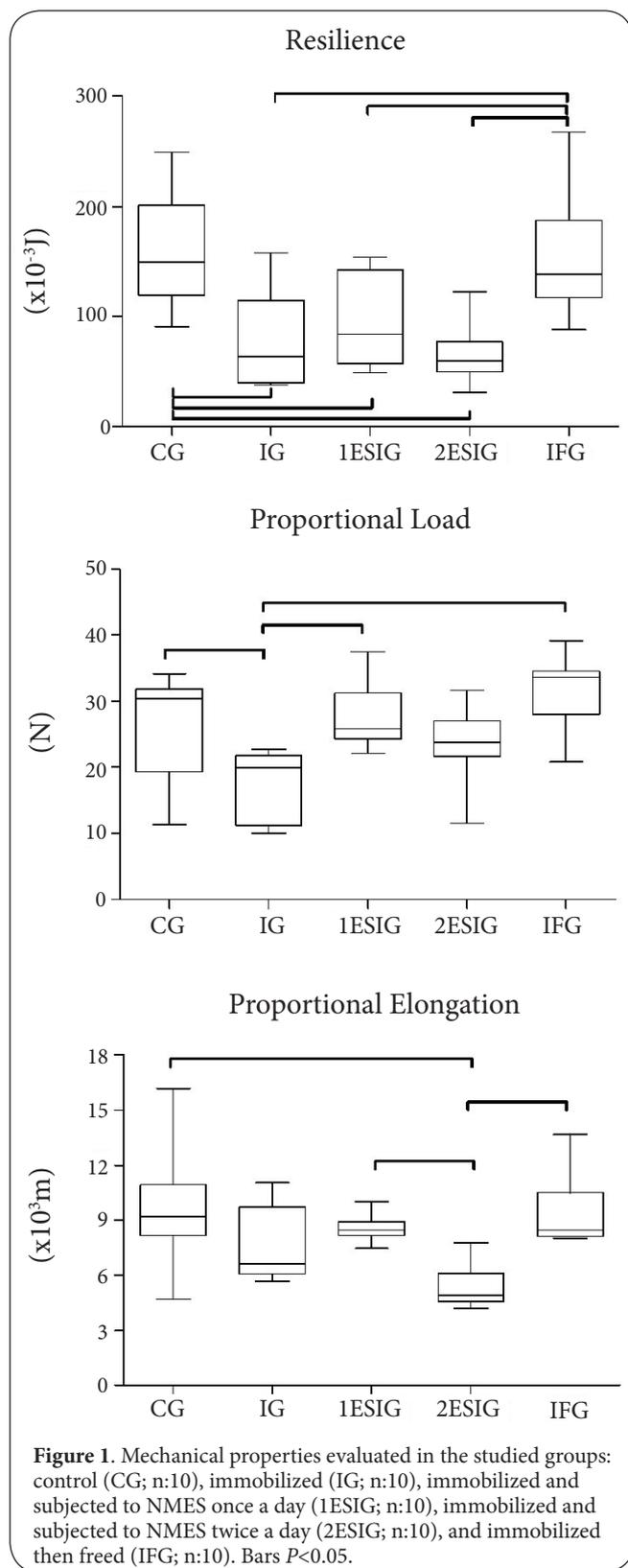
Mechanical properties

The boxplot in **Figure 1** indicates the results obtained from the analysis of the mechanical properties. Immobilization of the gastrocnemius muscle reduced the properties of Load at the Limit of Proportionality (LLP) (IG vs CG; $P<0.05$) and resilience (IG vs CG; $P<0.05$) compared to CG, but the property of elongation at the limit of proportionality remained unchanged (IG vs CG; $P>0.05$).

Free mobilization was able to restore the Resilience and LLP properties compared to the immobilized muscle (IFG vs IG; $P<0.05$). Free mobilization also showed better responses to the Resilience (IFG vs 1ESIG, 2ESIG; $P<0.05$) and Elongation at the Limit of Proportionality (ELP) (IFG vs 2ESIG; $P<0.05$) compared to the electrostimulated muscles. NMES applied once a day to the gastrocnemius muscle favored the reestablishment of the LLP (1ESIG vs CG; $P>0.05$; 1ESIG vs IG; $P<0.05$), whereas NMES applied twice a day presented values significantly lower to Resilience (2ESIG vs CG; IFG; $P<0.05$), and ELP (2ESIG vs CG; 1ESIG; IFG; $P<0.05$).

Morphology and morphometry

Qualitative analysis of the gastrocnemius muscle (HE) revealed that immobilization induced important signs of tissue abnormalities as necrosis and cell degeneration, hyaline necrosis and nuclear centralization, being the lateral portion of the gastrocnemius muscle most affected. The remaining groups submitted to immobilization showed necrosis and cell degeneration signs in an expressive way in both regions evaluated, however, these same groups showed regeneration of muscle fibers in the lateral portion. The medial portion of the IFG showed histological changes similar to those observed in the IG group, demonstrating increased amounts of nuclear centralization and necrosis and cell degeneration. The percentage of muscle fibers with morphological changes in each group are shown in **Table 1**, however, it is noted that less than five percent of the cells of the muscles evaluated were affected.



Fiber proportion

The percentages of fiber types and subtypes observed in

the medial portion of the gastrocnemius muscle of each experimental group (Table 2). The 1ESIG and the 2ESIG suffered significant reductions of TIIaF.

The percentage of type IIad fiber (TIIadF) was also reduced in the 1ESIG compared to the CG and the IFG ($P < 0.05$). Type IIc fiber (TIIcF) were significantly increased in the 1ESIG compared to the control and only immobilized groups (1ESIG vs CG; IG; $P < 0.05$). The remaining fibers, TIF, TIIIdF, TIIIdbF and TIIbF, did not differ significantly among the groups.

No significant differences in the percentage of TIF, TIIaF, TIIadF, TIIIdF, TIIIdbF and TIIbF in the lateral portion of the gastrocnemius muscle were observed among the groups. Only TIIcF was significantly increased in the 1ESIG compared to the IG and the IFG (Table 3).

Cross-sectional area

The results of the statistical analysis of the mean cross-sectional area of the medial gastrocnemius muscle fibers are presented in Figure 2. Simultaneous analysis of the variance indicated a significant difference only for TIF, TIIadF and TIIIdF (ANOVA; $P < 0.001$). Immobilization reduced the size of the cross-sectional area of TIF, TIIadF and TIIIdF (IG vs CG; $P < 0.001$). NMES applied once a day was able to reestablish the cross-sectional area of TIF and TIIIdF (1ESIG vs CG; $P > 0.05$) and produced values higher than those of the IG (1ESIG vs IG; $P < 0.001$). When NMES was applied twice a day, the section area was not reestablished for TIF, TIIadF and TIIIdF (2ESIG vs CG; $P < 0.001$). The 1ESIG showed a considerable increase in the section area of TIIbF. These fibers were not detected in the control animals but were present in the IG. The same increase was observed in the 2ESIG. Free remobilization was able to reestablish the values of the section areas only for TIIadF (IFG vs IG; $P < 0.001$), with a significant reduction being observed in TIIIdF compared to the CG (IFG vs CG; $P < 0.001$) (see Figure 2).

For the lateral portion of the gastrocnemius muscle, only TIF, TIIadF and TIIIdF were analyzed, but no difference was detected among groups ($P < 0.05$).

Intramuscular connective tissues

The mean quantities of intramuscular connective tissues of

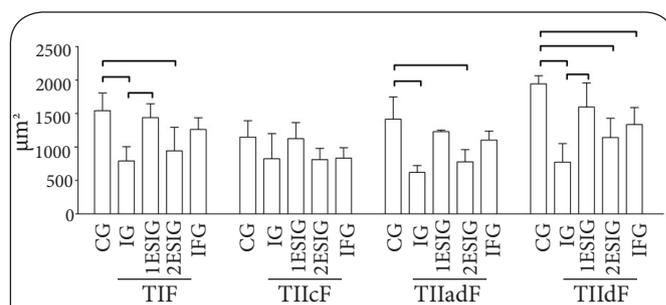


Figure 2. The cross-sectional areas (μm^2) of the medial gastrocnemius muscle fibers of the studied groups. FTI; FTIIad; FTIIId. Bars $P < 0.05$.

Table 1. Animals percentage with morphological alterations in the lateral (LG) and medial (MG) gastrocnemius muscles in the studied groups.

Alterations (%)	Groups									
	CG*		IG*		1ESIG*		2ESIG*		IFG*	
	MG	LG	MG	LG	MG	LG	MG	LG	MG	LG
Nuclear centralization	33	33	83	66	16	66	50	33	83	66
Lobulated fibers	--	33	66	83	16	33	33	--	33	--
Hyaline necrosis	--	66	33	83	--	--	33	--	33	33
Necrosis and cell degeneration	16	66	83	100	66	100	100	100	83	100
Regeneration	16	16	33	50	16	66	33	50	--	50
Splitting	--	--	66	33	50	50	33	33	83	33
Central core	--	33	--	66	16	--	50	16	16	66
Basophilic halo	16	83	--	66	--	50	16	33	83	16

*Less than five percent of the cells were identified per evaluated field; -Not detected

Table 2. Media±SD of the percentages of fiber types observed in the medial portion of the gastrocnemius muscle in the groups: Control (CG); Immobilized (IG); Immobilized and subjected to NMES once a day (1ESIG), and Immobilized and subjected to NMES twice a day (2ESIG); Immobilized and then free (IFG).

Groups	Type fibers						
	I	IIC [▲]	Ia	IId [▲]	IId [▲]	IIdb	Iib
CG	15.49±4.38	1.38±3.04	17.30±4.54	12.69±5.59	52.80±5.68	--	0.33±0.81
IG	15.64±6.43	1.09±0.95	13.19±7.61	6.70±3.29	62.92±12.36	--	0.46±0.47
1ESIG	15.28±9.87	11.01±4.63 ^{*,Δ}	--	2.13±3.47 ^{*,#}	68.85±11.41	1.78±4.35	0.96±1.31
2ESIG	16.32±6.56	7.99±4.36	--	7.99±6.55	66.60±4.28	--	1.09±1.26
IFG	22.51±5.92	3.52±2.78	7.18±4.63	12.22±2.09	54.21±7.17	0.36±0.89	--

[▲]Kruskal-Wallis Test ($P<0.05$); Dunnet Test-^{*}versus CG; ^Δversus IG; [#]versus IFG ($P<0.05$); --Not detected

Table 3. Media±SD of the percentages of fiber types observed in the lateral portion of the gastrocnemius muscle in the groups: Control (CG); Immobilized (IG); Immobilized and subjected to NMES once a day (1ESIG), and Immobilized and subjected to NMES twice a day (2ESIG); Immobilized and then free (IFG).

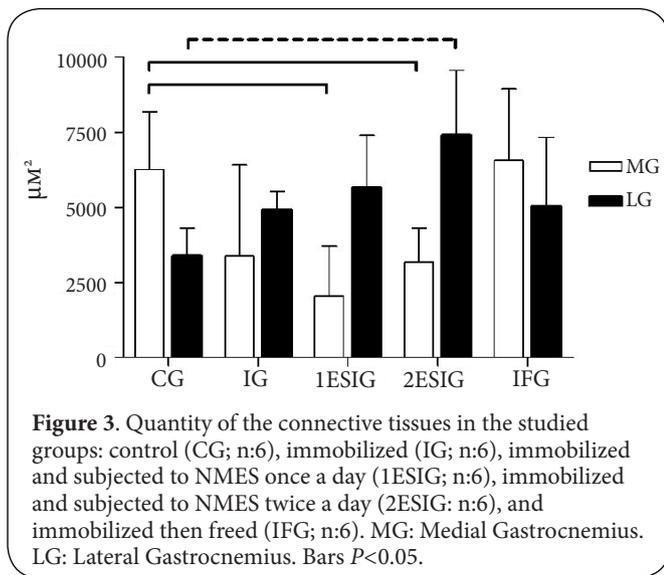
Groups	Type fibers						
	I	IIC [▲]	Ia	IId	IId	IIdb	Iib
CG	21.47±6.29	1.29±0.63	6.65±8.49	15.55±8.96	55.70±6.46	--	--
IG	19.69±5.55	--	2.23±2.33	12.40±9.05	61.84±28.38	1.23±2.08	--
1ESIG	18.86±13.18	15.34±9.03 ^{*,#}	6.77±14.25	4.71±5.59	54.32±19.78	--	--
2ESIG	21.06±12.29	4.74±5.95	--	7.57±8.34	60.90±21.29	1.15±1.56	4.58±5.85
IFG	31.76±14.09	1.24±0.80	3.01±4.67	16.70±9.89	47.72±31.53	--	--

[▲]Kruskal-Wallis test ($P<0.05$); Dunnet test: ^{*}versus CG; [#]versus IFG ($P<0.05$); --Not detected

each group are illustrated in **Figure 3**. For the medial portion of the muscle, only the 1ESIG and the 2ESIG presented mean connective tissue values significantly lower than those of the CG (CG vs 1ESIG; 2ESIG; $P<0.05$). In the lateral portion of the muscle, only the quantity of the connective tissues increased in the 2ESIG compared to the CG ($P<0.05$).

Discussion

The immobilization reduced the values of the LLP and the Resilience mechanical properties as well as promoted increased numbers of fibers presenting necrosis/degeneration, with greater expression in the lateral portion of the muscle. The medial portion of the muscle showed atrophy of the TIF,



TIIadF and TIIIdF, reduction of the TIIcF and TIIad numbers and no change in the amount of the intramuscular connective tissues. A previous study carried out in the laboratory showed that two weeks of immobilization in a shortened position atrophied TIF and TIIF [10], a fact that seems to be associated with an increase in proteolysis rather than with a decrease in protein synthesis [20]. This disequilibrium, in its turn, causes a reduction of the muscle volume, contraction force and amount of sarcomeres in series, especially when the muscle is maintained in shortened positions [21]. The detected changes in the mechanical properties must be associated with the reduced capacity of elongating in the elastic and plastic limits [12], as well as with the reduced stiffness and resilience. Clinically in humans, the knee immobilizing in extension promotes a reduction in the section of the quadriceps muscle area associated with increased in the relative mRNA expression of Myogenin, Myostatin, MyoD, MAFbx, P70S6K, LAT1/SLC, PAT1 and associated occurs a strength muscle decrease in 9% after five days of immobilization [22].

When NMES was applied once a day after the period of immobilization, LLP property returned the control patterns, however this did not occur when the technique was applied twice a day. An interesting observation was the reduced capacity for energy absorption in the elastic phase of 2ESIG, which presented the lowest value compared to the 1ESIG and IFG. Parallel findings obtained in the present study may justify these results.

The intramuscular connective tissues are responsible for the changes of the passive tension in the muscle [23], so that their cells can identify the magnitude, the mode and the direction of the mechanical force applied [24]. The adopted immobilization procedure allowed that the muscle stays shortened, however it is believed that the lateral and medial portions of the muscle responded to the immobilization period in a

different way. Such a result can be explained by the difference in muscle fascicle lengths among the different portions of the sural triceps. Kawakami et al., [25] reported that the articular angles and the internal as well as external fascicle designs in human beings can be different in the different portions of the gastrocnemius muscles. Moreover, it is believed that the lateral portion during (IG) and after (IFG) the immobilization period could have presented reduction in its contraction activity, knowing that while shortening the sural triceps a reduction in the muscle's lateral portion's activation occurs in human beings which would explain the increase in the conjunctive tissue [26]. However, analyzing the electrostimulated groups it is evident that the NMES procedure could have been concentrated in one of the muscle's portions, knowing that it was performed using only one electrode for both regions. In an experimental model of disuse for suspension, it was demonstrated that NMES applied twice a day (sine wave, 100 Hz, 500 ms) promotes the reduction in the speed of atrophy of the soleus and gastrocnemius muscles in both deep and superficial layers [27]. In humans, NMES applied twice a day (biphasic symmetrical rectangular-wave pulses, 100Hz, 400μs) for five days of immobilization promotes increased fiber type II CSA, associated with maintenance of the relative mRNA expression and p70S6K and myostatin reduction of MAFbx, MyoD and Myogenin, but did not increase the capacity of generate strength [22].

The data obtained revealed an increase in the quantity of the connective tissue in the lateral gastrocnemius muscle and the reduction of the same tissue in the medial region of the muscle groups 1ESIG and 2ESIG may have been responsible for the resilience alterations. Both NMES protocols accentuated responses derived from the period of immobilization. It is believed that it could have occurred because of the increase in the expression of the mechanical growth factor (MGF) and the connective tissues growth factor (CTGF), thus activating signaling cascades that resulted in the increased expression of collagen I and III in the muscle [6], which contributes to changes in the resilience in these groups.

Another fact that explains the reduced resilience obtained in the 2ESIG is the atrophy of TIF, TIIadF and TIIIdF observed in the gastrocnemius muscle's medial portion after the application of NMES twice a day. The quantitative data for the 1ESIG indicate a return of TIF and TIIIdF to the control conditions, what would justify responses proportional load and elongation. Studies using NMES once a day during [11,28] and after periods of immobilization [10], or applying it to healthy animals have detected an increase in the cross-sectional area (CSA) of the muscle fibers with a predominance of TIF and TIIF in the gastrocnemius muscle [10,11,28]. Data regarding the proportions of muscle fibers seem to indicate that NMES applied once a day increased the percentage of TIIcF in both portions of the gastrocnemius muscle, on the other hand reduced the TIIaF quantity. This was not observed in animals freely moving in their cages.

Another fact that stood out was the reduction of the TIIaF, since the presence of these fibers in the electrostimulated muscles was not detected. Associated with this, there was an increase in the number of TIIcFs in the groups submitted to the NMES. Moreover, the TIIdbF were present in the medial portion of the 1ESIG and lateral portion of the 2ESIG. These results suggest that the transitions were in the opposite directions from the TIIaF↔TIIbF↔TIIdbF↔TIIcF↔TIIadF↔TIIaF↔TIIcF↔TIF. However, when the NMES was applied for a longer period than used in this study, with a low frequency (10 Hz), the fibers to be first recruited were the TIIcFs, which underwent transition in one-way, being in the TIIcF↔TIIadF↔TIIaF↔TIF direction [28]. Thus, it can be seen that, regardless of the applied time of NMES, the initial recruitment involved the TIIcF fibers [30], even though a fiber transition occurs later. The findings in our study suggest that this transitional fiber event appears to be related to the expression of myostatin since an increase in IIb fibers together with a reduction of TIIaF and TIIcF can be observed in the myostatin-null (-/-) rats [27], data not evaluated in the study. Another observation regarding the TIIaF reduction would be the limitation of the fiber type (classifying) technique which could disguise its classification, nevertheless all methodological care as followed as described in other studies [18,19].

Free remobilization allowed the reestablishment of all mechanical properties, and the morphology of this group demonstrated the presence animals with changes such as centralized nuclei, necrosis/degeneration, splitting and basophilic haloes in a few fibers. The results indicate that the IFG animals presented responses similar to those of the 1ESIG, suggesting that the release model adopted can contribute to muscle stretching after the period of immobilization. Using a similar protocol, Mattiello-Sverzut et al., [10] showed that the release induced only irregularities in fiber contour.

Clinically, once daily NMES (asymmetric biphasic pulsed, 8.9Hz, 300µs, 3hours/days, For six weeks) promotes gains in muscle strength in fractured patients [27]. Patients submitted to hip arthroplasty and treated with NMES (biphasic current, 40Hz, 300µs, 1hour/day, For twelve days), also benefit because they increase walking-speed and reduce the time to Stair-climbing and sit-to-stand [29].

However, when the same resource is used only for five consecutive days during the period of immobilization and applied twice a day, is not capable of promoting significant increase in the capacity to generate muscular strength [22]. As reported, even with satisfactory clinical results, there are different patterns of NMES application, with controversial results. In this study the capacity to generate force during muscle contraction has not been evaluated, which is a limitation. However, there was post-immobilization NMES can produce effects on similarly elastic phase, regardless of the number of uses a single day.

Thus further studies are needed to identify how many times a day would be suitable for the best muscle response

after periods of immobilization, as well as the ideal frequency ranges for each muscle composition to contribute to the functional recovery in a shorter time.

Conclusion

On the basis of the protocols applied in the present study, we conclude that the muscle remobilization intensity can, either positively or negatively, influence the recovery responses post-immobilization, for as it was observed, once daily neuromuscular electrical stimulation (NMES) can recover most mechanical parameters (except resilience) and structural parameters of gastrocnemius muscle after casting, whereas twice daily NMES promoted more significant deleterious effects to muscles. Associate, the free movement imposed to animals, allowed them to identify their own mobilization limits, which did not cause mechanical nor structural overload in the muscles evaluated.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	LC	JP	FF	NP	AS	AM
Research concept and design	✓	--	--	--	✓	✓
Collection and/or assembly of data	✓	✓	✓	✓	--	--
Data analysis and interpretation	✓	✓	--	--	--	✓
Writing the article	✓	✓	--	--	--	✓
Critical revision of the article	✓	--	--	--	--	--
Final approval of article	✓	✓	✓	✓	✓	✓
Statistical analysis	✓	--	--	--	--	--

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