EFFECTS OF NEUROMUSCULAR ELECTRIC STIMULATION ON RATS' SOLEUS MUSCLE: A MORPHOMETRIC AND METABOLIC ANALYSIS

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SUMMARY

The aim of this work was to evaluate the effect of phasic neuromuscular electric stimulation (ES) on morphometric and metabolic parameters of rats' soleus muscles for 3, 7and 15 days. Wistar rats were divided into four groups (n=5): control (C), ES for 3 days (ES-3), ES for 7 days (ES-7) and ES for 15 days (ES-15). Glycogen content, muscle mass, fibers area and area fraction of the intramuscular connective tissue were assessed. The statistical analysis was performed by ANOVA and Tukey ($p<0.05$). Regarding muscle mass, there was a significant increase in ES-15 (11.55%) compared to C. The glycogen content didn't show significant changes in ES-3 when compared to C. ES-7 and ES-15 showed the significant increase of 74.19% and 80.64%, respectively, compared to C. In the morphometric analysis, a significant increase in ES-15 (16.23%) compared to C was found. The area fraction of the intramuscular connective tissue didn't present significant changes in all groups submitted to ES when compared to C. The ES fostered an increase of the glycogen content in 7 and 15 days, as well as increased muscle mass, fibers area and glycogen content in 15 days.

Keywords: *Electric stimulation therapy; Rats Wistar; Morphology; Metabolism; Physiotherapy.*

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INTRODUCTION

Muscular strength programs are important procedures and widely used in clinical physiotherapy. In addition to rehabilitation, there are other objectives to produce muscular hypertrophy, such as the esthetic factor, as well as for achieving a better sports performance.

Neuromuscular electric stimulation (NMES) became an important resource for producing muscle hypertrophy, especially after the reports published by the Russian doctor Yakov Kots in 1977 *apud* Kramer et al.[1] stating that NMES could produce strength gain in high-performance athletes up to 30-40% above those produced by maximum voluntary contraction of the muscle.

Although their experimental protocols were not well documented and its results could never been reproduced in the Western world, their reports contributed to increase the number of studies addressing the correlation between NMES and muscular strengthening. Since then, the developed studies seem to support the statement that this resource could strengthen muscles, both in healthy individuals[2,3] and in those submitted to muscle disuse regimens[3,4].

In the realm of experiments on animals, most of the studies involving NMES used chronic protocols, i.e., for long periods of time, as well as implanted electrodes. These studies showed that the resource predisposes a change of the fast fibers (IIB) to slow (IIA and I), increased resistance to fatigue with reduced tetanic tension, increased capillary density and perfusion, as well as an increased amount of myoglobins and satellite cells[5,6].

Furthermore, an increased contractile activity, whether by regular physical activity or by NMES, promotes the translation of a transporters population (GLUT4) insensitive to insulin, improving glucose uptake by muscles[7].

Despite of the several studies addressing NMES chronically applied, literature articles are scarce concerning its application during short periods (phased). In this context, Noronha et al.[8] did not find changes on fiber types, as well as anterior tibial muscle hypertrophy on rats stimulated on alternate days with surface electrodes (6 minutes, 3x/week, 8 weeks). On the other hand, a significant increase of the myofibrillary and sacroplasmatic protein synthesis evaluated 3 hours after stimulation (100 Hz, T_{ON}: 3s, T_{OFF}: 10s, implanted electrodes, for 20 minutes) on rats' soleus muscle and long extensor of fingers[9].

Although there are some animal studies indicating that NMES can produce strengthening, muscular hypertrophy and metabolic changes, the kind of stimulation employed may constitute a clinical issue, making unfeasible its application in human beings, once these studies used chronic NMES protocols (weeks or even months) and/or implanted electrodes.

Based on the scarcity of studies on animals indicating structural and metabolic changes by means of the NMES, mimicking the parameters used in human beings, the objective of this study was to assess the effects of phased NMES on morphometric and metabolic parameters on the soleus muscle of rats for 3, 7 and 15 days.

MATERIALS AND METHODS

Wistar rats (3-4 months old, 250-300g) were kept under controlled animal lab environment, receiving water and ration *ad libitum* and treated according to the guidelines of the Guide for Care Use of Laboratory Animals[10]. The study was approved by the committee of ethics in animal experimentation of the Federal University of São Carlos (protocol 010/2006).

The animals were divided into 4 groups (n=5): control (C), electrical stimulation for 3 days (NMES-3), 7 days (NMES-7) and 15 days (NMES-15).

Study conducted at the Metodista University of Piracicaba, SP - Brazil.

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Following anesthesia with sodium pentobarbital (50 mg/Kg of body weight), the left posterior paw was trichotomized and the NMES was performed as a daily session of 20 minutes, for a period of 3, 7 and 15 days. An electrode was placed on the inguinal region and the other on the sural triceps muscle. The electric stimulation parameters were the following: 10 Hz frequency, 0.4 ms phase, and quadratic 2-phased pulse. Current power was standardized at 5.0 mA, from the visualization of muscle contraction, being added by 1.0 mA at each 5 minutes, aiming to keep an effective contraction during the whole session period. The silicone-carbon electrodes had an area of 1cm^2 each.

After the experimental period, we conducted the following analyses: glycogen content on the soleus muscle, as well as mass, fibers area and density of intramuscular connective tissue area.

For determining muscular glycogen, we adopted the recommendation described by Lo et al.[11], which consists of the digestion of muscular samples in 30% KOH at 100° C and the precipitated glycogen from passing through ethanol. Between one and another precipitation phase, the sample was centrifuged at 3000 rpm (rotations per minute) for 15 minutes and the precipitated glycogen was submitted to acid hydrolysis in the presence of phenol. The values were expressed as mg/100mg of wet weight.

For the morphometric analysis of the soleus muscle, its ventral segment was fixated into buffered formalin solution and the material was processed into paraffin, obtaining several 7μm-wide non-serial cross sections, which were stained with Hematoxilyn-Eosyn (HE). An image analysis system constituted of an Image Pró-plus 4.0 (Media Cybernects) software, digital camera (JVC) attached to a microscope (Zeiss) integrated to a computer was employed. all images were captured with an objective lens with 10x magnification. Areas of 375 fibers were assessed on each animal, selected as follows: 15 fibers/ area, being 5 areas for each section, totaling 5 sections per animal. A checkered reticulum was used for the selection of 15 fibers per section, randomly, which matched the intersections of the segments.

For assessing intramuscular connective tissue density, the planimetry system by points count [12] and the quantification were calculated by means of a reticulum with 2500μm^2 squares containing 56 segment intersections. The matching points were counted on the endomysium and perimysium, on 5 areas per section, being 5 sections for each animal, totaling 1400 points per animal. The area concerned to connective tissue (area density) was calculated by dividing the sum of matching points on segment intersections on the connective tissue (endomysium and perimysium) by the total number of points.

The statistical analysis was initially provided by Kolmogorov-Smirnov's normality test and by the homocedasticity test (Bartlett's criterion). Once variables were in accordance to the parametric methodology, ANOVA and the F test were used, and, when a significant difference was found, the Tukey HSD test was applied for multiple comparisons. For all calculations, a significance level of 5% was established.

RESULTADOS

Concerning mass, we didn't find significant changes on groups NMES-3 and NMES-7 when compared to C; however, an increase ($p<0.05$) of 11.55% was seen compared to C. In addition, NMES-15 showed increase ($p<0.05$) compared to NMES-3, and NMES-7, of 9.53% and 8.93%, respectively, but NMES-3 did not differ from NMES-7 significantly.

Muscular glycogen content showed no significant changes on NMES-3 when compared to C. But the NMES-7 and the NMES-15 showed an increase ($p<0.05$) of 74.19% and 80.64%, respectively , when compared to C, evidencing that they did not differ significantly from each other.

In the morphometric analysis, Groups NMES-3 and NMES-7 showed no significant changes on muscular fibers area when compared to C, while NMES-15 showed an increase ($p<0.05$) of 16.23% compared to C. In addition, the NMES-15 showed an increase ($p<0.05$) of 20.78% when compared to NMES-3, and of 14.94% compared to NMES-7, evidencing that NMES-3 showed no significant difference from NMES-7.

Intramuscular connective tissue density showed no significant changes on all groups submitted to NMES when compared to C, as well as to the groups submitted to intervention. All values can be seen on Table 1 and Figure 1.

	C	NMES-3	NMES-7	NMES-15
Mass (mg)	124.6±5	126.9±5.1	127.6±3.9	139±4.8*†#
Area (µm2)	2574±560	2478±351	2603±350	2992±273*†#
Connective (%)	8.82±3.55	7.78±3.47	9.33±4.95	8.97±5.12
Glycogen (mg/100mg)	0.31±0.03	0.36±0.05	0.54 ± 0.04*#	0.56±0.03*†#

Table 1 - *Average±msd of soleus muscle mass (mg), muscular fibers area (µm2), connective tissue area density (%), glycogen content (mg/100mg) of the control (C), NMES for 3 days (NMES-3), 7 days (NMES-7) and 15 days (NMES-15) groups. n=5, p<0.05, * compared to control, † compared to NMES-7, # compared to NMES-3.*

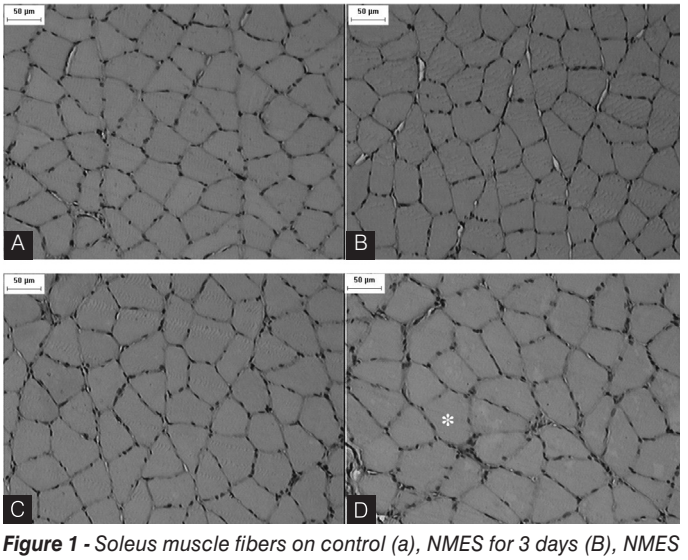

Figure 1 - *Soleus muscle fibers on control (a), NMES for 3 days (B), NMES for 7 days (C) and NMES for 15 days (D) groups. Note the increased area of muscular fibers (asterisk, compared to control group).*

DISCUSSION

Several studies have been conducted showing the effectiveness of NMES in promoting changes to the musculoskeletal system, such as muscular fibers transition, increased resistance to fatigue, capillaries density and perfusion, myoglobin content, satellite cells, glucose uptake, as well as reduces tetanic tension [5,6]. However, the way in which the stimulus is provided in these studies does not mimic clinical practice, since they included protocols with implanted

electrodes for a period of weeks or even months, making its application in human beings unfeasible.

In the present study, the protocol with short application periods and surface electrodes are consistent to clinical practice. In this experimental environment, NMES was shown to promote increased glycogen content on the soleus muscle on the seventh day, as well as increased mass, fibers area and glycogen on the fifteenth day. This fact demonstrates the correlation of contractile activity with power homeostasis and muscular fiber morphology, pointing out to a muscular hypertrophy picture.

Some studies showed similar results, but using chronic stimulation and/ or implanted electrodes. Egginton and Hudlick[13] using NMES (10 Hz, 8 hours/ day) for 3 days in rats, applied with an implanted electrode on fibular nerve, found a 12% increase of anterior tibial muscle mass, as well as increased blood flow.

In line with that, Atherton et al.[9] showed that the resource, used at a frequency of 100 Hz, T_{ON} 3s, T_{OFF} 10s, for 20 minutes with implanted electrodes promoted a significant increase of myofibrillary and sarcoplasmatic proteins assessed after 3 hours of stimulation on rats'soleus muscle and long extensor of the fingers.

Despite of the scarcity of studies addressing NMES for short periods, as well as surface electrodes, Noronha et al.[12] assessed the effects of NMES on rats' anterior tibial muscle (frequency of 52 Hz, T_{ON} 10s, T_{OFF} 10s and rise of 2s), after 8 weeks, 3 times a week, with 20 contractions in each session, in alternate days, respecting the weekends. The authors did not see muscular hypertrophy or changes on the types of fibers.

The results presented here differ from those found by Noronha et al.[12], although both studies used NMES for short periods and surface electrodes. This fact is justified by the difference of current parameters, as well as by the kind of contraction electrically elicited. Probably, the large number of non-tetanic contractions (12000/ session) was enough to promote morphologic and metabolic changes on soleus muscle, a fact that has not been found in that study, which used a total of 480 tetanic contractions of 10 seconds each. Another difference is on the frequency employed, since the 10 Hz frequency selectively depolarizes type-I muscle fibers' motoneurons.

Muscular hypertrophy found in this study, characterized by an increased muscle mass and soleum fibers area during 15 days of NMES can be explained by the fact that this stimulus triggers a cascade of events which characterizes on the transduction of a still unknown signal that regulate the expression of specific muscle growth factors, such as IGF-1, mechanical growth factor (MGF), as well as myostatin. Thus, some studies report that NMES promotes an increased intramuscular proteins synthesis, resulting in muscle hypertrophy [9,14,15].

The importance of studies evaluating glycogen content on the muscle is based on direct correlations between those supplies and the aerobic power or performance of the body, so that changes on enzymes profile and on glycogenic supplies are responsible for a reduced muscular effectiveness, as well as the development of exhaustion state [16].

In this context, NMES has also promoted a rise on glycogen supplies in 7 and 15 days. This can be justified by the increased glucose uptake by GLUT4 population insensitive to insulin that is externalized, being also a result of the activation of cytosolic enzymatic systems on glucogenesis [17]. Clearly, NMES promotes an increased contractile activity on muscular fibers, thus glucose uptake and metabolism dynamics and cellular metabolic paths activity are increased [18].

Etgen et al.[7] assessed GLUT4 content on plantar muscles of rats after chronic NMES, and found an increase of 82%. Longer NMES periods (30-40 and 60-90 days) showed only a trend towards an increased GLUT4 content, reaching a plateau around the 30th and 40th days. An important result of the study by Hamada et al.[19] was that body glucose uptake in rats is strongly increased as a response of NMES applied for 20 minutes, with such increase lasting for at least 90 minutes after the resource use is completed.

Although the analyses of this study show both morphologic and metabolic changes, no functional assessments were made on soleus muscle such as, for example, muscular strength, to check the existence or not of any change on the recruitment pattern of motor units which, in turn, can also cause bias to contractile force.

Furthermore, some considerations must be made concerning the various researches showing failures on the description of methodological parameters used on NMES. For Robison and Snyder[20], there are failures on the descriptions of the methods, since many studies do not extensively record important experimental details, such as training parameters and physical characteristics of the current. However, in the last few years, a stronger control has been provided to variables and a better evenness of the research methods. Thus, standardizing studies involving NMES became critical in order to enable reproducibility, as well as comparisons between various studies.

CONCLUSION

NMES promoted an increase of the glycogen supplies on rats' soleus muscle during 7 days, as well as an increased muscular mass, fibers area, and glycogen supplies during the period of 15 days. Therefore, the importance of intervention with NMES is highlighted, aiming to promote muscular hypertrophy, showing the correlation between contractile activity and power homeostasis, as well as fiber's morphology.

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