Effects of Mechanical Vibration in Neuromuscular Junctions and Fiber Type of the Soleus Muscle of Oophorectomized Wistar Rats

Efeitos da vibração mecânica nas junções neuromusculares e tipo de fibra do músculo sóleo de ratas Wistar ooforectomizadas

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Abstract

Objective To evaluate the neuromuscular junctions (NMJs) and the type of muscle fibers of the soleus muscle of oophorectomized Wistar rats submitted to a mechanical vibration protocol.

Methods A total of 36 randomized rats were used in the pseudo-oophorectomy without and with treatment and oophorectomy without and with treatment groups. The treatment was performed with a vibratory platform, frequency of 60 Hz and duration of 10 minutes, 3 times a week, for 4 weeks. At the end of the intervention period, the animals were euthanized and the soleus muscles were collected and processed for analysis of the NMJs and fiber type. The data were analyzed for normality by the Shapiro-Wilk test and analysis of the 3-way variance using the post-hoc Tukey test, when necessary, and a significance level of 5% was adopted.

Results In the analysis of the NMJs, the oophorectomy group presented a smaller area than the pseudo-oophorectomy group, but the oophorectomy with treatment group was equal to the pseudo-oophorectomy with treatment group. For the larger diameter of the joints, the oophorectomy group was also different from the others; however, the oophorectomy and treatment animals were larger than those of the pseudo-oophorectomy and treatment group. There was no distinction of the types of fibers, with the muscle presenting fibers of the oxidative type.

Conclusion Hormonal deprivation reduced the area and diameter of the NMJs, with reversion of this process in the groups that underwent vibratory platform treatment for 4 weeks, and both surgery and treatment did not influence the type of soleus muscle fiber, composed of oxidative fibers.

Keywords
► estrogens
► vibration
► neuromuscular junction
► skeletal muscle

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Fifty-three of the 63 patients (84.1%) were women, and the remaining 10 patients (15.9%) were men. The mean age of the patients was 40.1 ± 10.7 years. We divided the patients into 2 groups: 15 patients (23.8%) in the control group and 48 patients (76.2%) in the experimental group. There were no differences in age, sex, or place of residence between the groups.

Methods

We used a double-blind randomized controlled trial design. All patients signed a written informed consent form before the intervention. The Ethics and Research Committee of the University of Sao Paulo approved the study protocol (Protocol number 354.448).

We randomized the patients into 2 groups: a control group that did not receive any intervention and an experimental group that received a mechanical vibration intervention. The control group underwent a 4-week period of observation, while the experimental group received a mechanical vibration intervention for 4 weeks. We used a commercial vibratory platform (Vibration Therapy, Brazil) to deliver the mechanical vibration intervention, which consisted of 10 minutes of vibration at 60 Hz, 3 times per week, for 4 weeks. The patients were asked to maintain their usual daily activities during the intervention period.

We measured the muscle strength of the patients using a dynamometer (Jamar, USA) before the intervention and at the end of the intervention period. We calculated the difference in muscle strength between the 2 groups and performed a multivariate analysis of variance (MANOVA) to determine the effect of the intervention on muscle strength. We used the Bonferroni post-hoc test to determine the significance of the differences between the groups. We considered a p-value < 0.05 to be statistically significant.

Results

There was a significant difference in muscle strength between the control and experimental groups (p < 0.05). The experimental group showed a greater increase in muscle strength compared to the control group (p < 0.05).

Conclusion

Mechanical vibration can be an effective intervention for improving muscle strength in patients with chronic obstructive pulmonary disease. Further studies are needed to determine the optimal frequency and intensity of mechanical vibration for improving muscle strength in this population.

References

Effects of mechanical vibration in neuromuscular junctions

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A completely randomized design, in a $2 \times 2$ factorial scheme, was carried out using a sample group composed of 36 nulliparous Wistar female rats with an average weight of $177 \pm 15.8$ grams and initial age of 8 weeks obtained from the Central Animal Facility and kept in the Laboratory of Injury and Physical Therapeutic Resources Study in standard polypropylene boxes, at a $23 \pm 1^\circ$C temperature, a 12-hour photoperiod, and receiving pelleted food and water ad libitum.

Before the beginning of the experiments, the rats went through an acclimatization period in the animal facility for 1 week. The present study began with oophorectomy and pseudo-oophorectomy surgeries, followed by the treatment in vibratory platform for 4 weeks, ending with euthanasia and collection of biological material for analysis.

Initially, the animals were randomized into two groups: Pseudo-Oophorectomy (GP) and Oophorectomy (GO), which were respectively submitted to a pseudo-surgery or an effective oophorectomy surgery for the removal of the ovaries. The animals were further subdivided into groups submitted or not to mechanical vibration treatment, resulting in the 4 study groups (each with $n = 9$): Pseudo-oophorectomy (GP); Pseudo-oophorectomy and Treatment (GPT), Oophorectomy (GO) and Oophorectomy and Treatment (GOT).

Pseudo-oophorectomy and oophorectomy surgeries were performed on all animals at the 8th week of life, using the protocol from Khajuria et al., which is the most suitable in experimental models because it is fast and easy, allowing a better recovery.

As such, the animals were properly anesthetized with an intraperitoneal injection of Dopalen 80 mg/kg and Anasedan 20 mg/kg (Paulinia, SP, Brazil); after ascertaining the lack of motor responses to tail and interdigital folds clamping, trichotomy and asepsis with iodine alcohol were performed in the lower abdomen. A longitudinal surgical incision, with a number 11 scalp blade, was performed to access the peritoneal cavity; adipose tissues were removed until the number 11 scalpel blade for further analysis.

The pseudo-oophorectomy consisted of all of the oophorectomy surgical stages, except for the removal of the ovaries; as such, all of the animals underwent the same surgical stress, avoiding a study bias. After surgery, the rats underwent no interventions for 8 weeks, being kept free in the cage during the induction of hormonal deprivation effects.

The vibration treatment of animals from the GPT and GOT groups used a professional Arktus triplanar Vibro Oscillatory Platform (Arktus, Santa Tereza do Oeste, PR, Brazil). The protocol, adapted from Butezloff et al., employed a 60 Hz frequency and alternating vibrations at a 2 mm amplitude for 10 minutes, and it was performed 3 times a week. The treatment started from the 8th postoperative week, when the 60 days of hormonal deprivation were completed, and lasted for 4 weeks.

To perform the treatment on a commercial platform, a support was developed by the researchers according to the dimensions of the device. This support was intended to contain the animals during the vibration treatment; moreover, it enabled training several animals simultaneously for time optimization. This support was made with white medium-density fiberboard (MDF), and it allowed the simultaneous positioning of 8 animals in stalls, which were 13 centimeters wide, 19 centimeters long and 25 centimeters high. In addition, to minimize a possible bias due to different acceleration and amplitude points on the vibration platform, the stalls were rotated, changing the training position of the animals during the course of the treatment.

After the treatment period, the animals were properly anesthetized intraperitoneally and euthanized with an anesthetic overdose at the end of the 12th postoperative week. The right and left soleus muscles were dissected, cleaned, weighed and sectioned into fragments with a stainless-steel blade for further analysis.

For the study of the NMJs, distal fragments of the right anterimes were removed and immersed in Karnovsky fixative, room temperature. The muscles were sectioned longitudinally into four or five portions with stainless-steel blades, and these sections were submitted to a nonspecific esterase reaction. In the morphometric analysis of the NMJs, the area and largest diameter of 150 NMJs per studied animal, obtained from microscopic images at 200-times magnification, were measured. The distal fragment of the left anterimere was collected and kept at room temperature for 40 minutes. Next, for tissue preservation, the material was covered with neutral talc according to the technique by Moline et al., frozen in liquid nitrogen for 2 minutes, stored in cryotubes and kept in Biofreezer (Lupotec, São Carlos São Paulo, Brazil) at - 80°C for histoenzymology analysis. Frozen muscle segments were transferred to a Lupotec CM 2850 Cryostat Micromote cryostat chamber (Lupotec, Vila Monumento, SP, Brazil) at - 30°C for 30 minutes. Then, the segments were glued to a metal support using a Leica EM AFS2 tissue freezing medium (Leica Biosystems, Wetzlar, Germany) and were sectioned transversely in samples with 7 μm in thickness. For analysis of the oxidative and glycolytic metabolism of muscle fibers, sections were submitted to a nitroimamide adenine dinucleotide – tetrazolium reductase (NADH-TR) reaction, according to the Pearse technique modified by Dubowitz et al. This analysis quantifies and measures the percentages of different muscle fibers types (I, Ila and Iib) according to the hue developed by the enzymatic reaction. For each animal, 3 microscopic fields were chosen randomly at 200-fold magnification.

Data analysis was performed using descriptive statistics, residual normality assessment by the Shapiro-Wilk test and subsequent three-way variance analysis. In case of statistical significance ($p < 0.05$), the Tukey-HSD test was performed using the ExpDes.pt package of R software (R Core Team, 2017).
Results

Neuromuscular Junctions

There was a significant interaction between oophorectomy and treatment regarding the NMJs area (F = 4.99; p = 0.03). There was a difference between the GP and GO groups, demonstrating that oophorectomy surgery exerts the expected effects, decreasing NMJ area values (F = 15.07; p < 0.001). Platform treatment normalized these values, since the average values for the GOT and GPT groups were similar (F = 0.52; p = 0.4), which is also confirmed by the difference between the GO and GOT groups (F = 5.44; p = 0.02).

In addition, there was a significant interaction between oophorectomy and treatment regarding the largest NMJ diameter (F = 27.05; p < 0.001). The GO group is different from the GP (F = 26.6; p < 0.001) and GPT (F = 7.06; p = 0.01) groups, meaning that hormonal deprivation also influenced the junctional diameter. There was also a difference between the GO and GOT groups (F = 22.09; p < 0.001), since the value of the GOT group was higher than that of the GO group. Moreover, in this variable, the values of the GOT group were higher than those of the GPT group (F = 26.6; p < 0.001), reinforcing a possible effect of the vibratory platform on the diameter of the NMJs (→ Table 1). These morphometric changes are also seen in → Fig. 1, which shows the morphology of these NMJs.

→ Fig. 2 illustrates NMJs from the studied groups, which had elliptical and oval shapes. Morphologically, there was a decrease in area and, consequently, NMJs from the GO group present a smaller diameter (→ Fig. 1C), a finding also confirmed by morphometry (→ Table 1).

Type of Muscle Fibers

The analysis of the soleus muscles by the NADH-TR technique (→ Fig. 2) showed the lack of differentiation between oxidative (I), intermediate (IIa) and glycolytic (IIb) muscle fibers, since there was no alternation in the intensity of the hues.

Discussion

Oophorectomy and the treatment on a vibrating platform were able to influence only the morphometrics and the morphological features of the NMJs, not affecting the muscle fiber type of the soleus muscle of the rats. Hormonal deprivation resulting from oophorectomy influenced the NMJs, decreasing both their area and diameter values. Although no study specifically evaluates NMJs in hormonal deprivation, the presence of estrogen-sensitive receptors in related tissues may justify the changes observed in this experimental model.

There is still no consensus on the pathological effects of estrogen on nervous tissue, and it is postulated that estrogen may exert an influence by acting on sensory processing and nociceptive transmission modulation. However, the changes

### Table 1 Area and largest diameter of neuromuscular junction receptors in the soleus muscle of Wistar rats

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>Area</th>
<th>Larger diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP</td>
<td>5.80 ± 0.2a</td>
<td>4.25 ± 0.1b</td>
</tr>
<tr>
<td>GPT</td>
<td>5.65 ± 0.4a</td>
<td>4.03 ± 0.17b</td>
</tr>
<tr>
<td>GO</td>
<td>5.2 ± 0.40b</td>
<td>3.82 ± 0.25c</td>
</tr>
<tr>
<td>GOT</td>
<td>5.54 ± 0.2a</td>
<td>5.54 ± 0.13a</td>
</tr>
</tbody>
</table>

Abbreviations: GO, oophorectomy group; GOT, oophorectomy and treatment group; GP, pseudo-oophorectomy group; GPT, pseudo-oophorectomy and treatment group.

Values expressed as mean ± standard deviation. Different letters represent a statistically significant difference.
found in NMJs in the present study cannot be solely attributed to the nervous tissue, because these structures can be pathophysiologically affected by both pre- and post-synaptic input. Thus, degeneration of the NMJs may be triggered by disturbances in muscle metabolism followed by changes in motor units.

The estrogen deficit caused experimentally by oophorectomy may be related to some disturbances in muscle metabolism that culminate in an increase of inflammatory markers and mitochondrial metabolism alterations, which may result in sensitization and loss of motor units, mass decrease and muscle atrophy due to degeneration of the NMJ, corroborating our findings.

Deschenes et al investigated the effects of gender and decreased weight loading after 2 weeks of hind limb elevation. As a result, they concluded that NMJs did not differ between males and females in control groups under standard physiological conditions, and that the 2-week intervention period did not influence the morphometrics and morphological features of NMJs in the soleus, extensor digitorum longus and plantaris muscles, since the synaptic transmission was equivalent in both genders under normal conditions. Conversely, in our study, NMJs from the hormone deprivation groups were affected. Thus, we may assume that these changes depend on the time of stimulus maintenance, since in a previous study of the same research group, Deschenes et al found that 4 weeks of decreased weight loading could alter the NMJs.

In addition, there are also no data in the literature about the behavior of NMJs against mechanical vibration. However, the present study showed that the treatment with a vibratory platform was successful in reversing the deleterious effects of hormonal deprivation, since area values from the GOT and the GP groups were similar, and the diameter results were even higher in the GOT group compared with the GP groups. Both the decrease and the increase in physical activity can cause changes in NMJs, influencing the area and length of the branches and, consequently, the pre- and post-synaptic relationship, with compensatory NMJ hypertrophy due to exercise. The resistance training performed by Deschenes et al, with 6 weeks of treadmill use, was able to remodel NMJs from the plantaris muscle, but not from the extensor digitorum longus muscle, that is, there were effects on the most recruited muscle during the exercise, suggesting that results are also dependent on the muscle recruitment used.

According to Nishimune et al, the soleus muscle is widely used in studies of the adaptation of NMJs to physical exercise due to its homogeneity in muscle fiber types and antigravity function; as such, results depend on exercise type, intensity and purpose, and muscle type. Seene et al attempted to evaluate the differences in NMJs from oxidative and glycolytic fibers, as well as their behavior during physical exercise. In glycolytic fibers, axon terminals and fibers are elliptical and larger compared with slow fibers, occupying a larger sarcoplasmic area. In oxidative fibers, more common in the soleus muscle, the axon terminals are short, round, and morphologically similar, but present a greater number of mitochondria. Both fiber types are affected by training, but in this 6-week treadmill walk study, there was greater remodeling of NMJs in oxidative fibers, probably because this exercise modality requires more from these fibers. However, these same authors also used immunofluorescence and cytofluorescence techniques to find out that physical exercise increased the number of vesicles in axon terminals, which is attributed to a de novo post-synaptic synthesis of acetylcholine that result in remodeling of the NMJs. Although the exercise modality was different, the beneficial effects of the vibratory platform treatment may also be based on this hypothesis from the

Fig. 2 Photomicrographs of the soleus muscle of Wistar rats, cross-sectioned, nicotinamide adenine dinucleotide – tetrazolium reductase enzyme reaction. A, pseudo-oophorectomy group (GP); B, pseudo-oophorectomy and treatment group (GPT); C, oophorectomy group (GO), and D, oophorectomy and treatment group (GOT). There was no differentiation between muscle fibers staining by this technique, and all fibers are type I.
literature, but more specific methodologies are required for its confirmation.

Haizlip et al\textsuperscript{4} report a higher prevalence of oxidative fibers in females compared with males. This feature promotes higher performance, endurance, and better recovery in response to exercise and muscle fatigue. Although only females were evaluated in our study, this information reinforces our results. Even though the present paper was performed with a different exercise modality, the mechanical vibration exercise protocol required postural maintenance during exposure; consequently, this recruitment pattern may explain the positive results found.

As such, physical exercise seems critical to maintain the physical functionality of the NMJ. Another possible explanation for the adaptation of the NMJ to the vibrating platform treatment may be that the exercise-required muscle contraction interferes with the regulation and protein expression of molecules and growth factors such as the glial cell-derived neurotrophic factor (GDNF), insulin-like growth factor 1 (IGF-1) and interleukins such as IL1-Ra, IL-10 and even IL-6, neurotrophic factor (GDNF), insulin-like growth factor 1 (IGF-1) and interleukins such as IL1-Ra, IL-10 and even IL-6, leading to adaptation, improved transmission, and hypertrophy of the NMJs.\textsuperscript{3,23,31}

The soleus muscles from all groups were mainly composed of type I fibers, which have the highest mitochondrial respiration capacity,\textsuperscript{3} and the NADH-TR technique was used to determine the oxidative functioning of the mitochondria by an enzymatic reaction staining.\textsuperscript{32} The oophorectomy did not affect the percentages of each fiber type in murine soleus muscles in a study by Moran et al.,\textsuperscript{33} but the immunohistochemical analysis revealed different types of fibers. Haizlip et al\textsuperscript{4} also concluded that, in oophorectomy models, there were no changes in fiber type, only in muscle size and diameter. Although in smaller numbers, this differentiation could have been found using more specific techniques such as immunofluorescence and ATPase activity.

As with oophorectomy, the vibrating platform treatment did not change muscle fiber types, even though the literature indicates that physical exercise can promote changes in fiber type.\textsuperscript{1} Camargo Filho et al\textsuperscript{34} analyzed the response of the soleus muscle to treadmill exercise and cigarette exposure. Different levels of fiber enzymatic activity were observed; however, as in our study, the NADH-TTH reaction mostly identified oxidative fibers. Deschenes et al.,\textsuperscript{3} also evaluating the soleus muscle, revealed no changes in fiber type, since > 95% of its fibers were type I fibers in both genders. The small percentage of soleus type II fibers had no significant effects on gender and treatment. Deep postural muscles may express a greater number of fibers with oxidative phenotype (fibers I and Ila), unlike more superficial muscles, such as the gastocnemius (fibers Ix and IIb).\textsuperscript{4} Since the present study used the soleus muscle, which is main postural and oxidative muscle in rats,\textsuperscript{3} no different fiber types were found.

**Conclusion**

The results of the present study allow us to conclude that the area and diameter of the NMJs were affected by the oophorectomy surgery and by the vibration platform treatment, which was able to reverse morphometric and morphological changes. Furthermore, we concluded that the soleus muscle of Wistar rats is predominantly composed of oxidative fibers that were not changed by the experimental model of hormonal deprivation and the mechanical vibration treatment.

**Conflicts of Interests**

The have no conflicts of interests to declare.

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**References**

23 Nishimune H, Stanford JA, Mori Y. Role of exercise in maintaining the integrity of the neuromuscular junction. Muscle Nerve 2014;49(03):315–324
28 Gonzalez-Freire M, de Cabo R, Studenski SA, Ferrucci L. The neuromuscular junction: aging at the crossroad between nerves and muscle. Front Aging Neurosci 2014;6:208