


# 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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## Resumo

**Objetivo** Avaliar as junções neuromusculares (JNMs) e o tipo de fibras musculares do músculo sóleo de ratas Wistar ooforectomizadas e submetidas a um protocolo de vibração mecânica.

**Métodos** Foram utilizadas 36 ratas randomizadas nos grupos pseudo-ooforectomia sem e com tratamento e ooforectomia sem e com tratamento. O tratamento foi realizado com plataforma vibratória, frequência de 60 Hz e duração de 10 minutos, 3 vezes por semana, durante 4 semanas. Ao término do período de intervenção, os animais foram eutanasiados e os músculos sóleos coletados e processados para análise das JNMs e tipo de fibra. Os dados foram analisados quanto à normalidade pelo teste Shapiro-Wilk e análise da variância de 3 vias, utilizando o pós-teste de Tukey quando necessário, tendo sido adotado o nível de significância de 5%.

**Resultados** Na análise das JNMs, o grupo ooforectomia apresentou área menor que o grupo pseudo-ooforectomia, porém o grupo ooforectomia tratado igualou-se ao grupo pseudo-ooforectomia tratado. Para o maior diâmetro das junções, o grupo ooforectomia também se apresentou diferente dos demais; porém, os animais do grupo ooforectomia tratado foram maiores que o grupo pseudo-ooforectomia tratado. Não houve distinção dos tipos de fibras, com o músculo apresentando fibras do tipo oxidativo.

**Conclusão** A privação hormonal reduziu a área e o diâmetro das JNMs, com reversão deste processo nos grupos que realizaram o tratamento com plataforma vibratória durante 4 semanas e, ainda, tanto cirurgia quanto tratamento não influenciaram no tipo de fibra do músculo sóleo, composto por fibras oxidativas.

## Palavras-chave

- ▶ estrogênio
- ▶ vibração
- ▶ junção neuromuscular
- ▶ músculo esquelético

## Introduction

The neuromuscular junction (NMJ) allows communication between the peripheral nervous system and musculoskeletal fibers; it transmits electrical impulses from motor neurons to the connected myofibrils, which are responsible for the development of contractile force.<sup>1,2</sup> The NMJ is composed by the pre- and post-synaptic compartments. Its components include the peripheral axon, its myelin sheath and Schwann cells, acetylcholine receptors and vesicles, acetylcholinesterase and muscle basement membrane.<sup>3</sup>

Mammalian muscle fibers may be divided into oxidative (I), oxidative-glycolytic (IIa) and glycolytic (IIb and IIx) types according to their metabolic activity.<sup>4</sup> The NMJs depend on muscle fiber type, and in the soleus muscle, oxidative fibers are innervated by motor neurons with low conduction velocity, being able to sustain tension for longer periods.<sup>1</sup>

Since muscle tissue is plastic, its size and characteristics may change in response to stimuli.<sup>5,6</sup> Estrogen is a hormone present in both genders, but it is more characteristic of females. The musculoskeletal system also has estrogen receptors, and abnormal hormonal levels result in cellular effects, including apoptosis and protein and gene transcription, that consequently affect muscle structure, performance and strength.<sup>4</sup>

Another stimulus that affects muscle tissues is physical exercise, which promotes changes both in fiber type, leading to fiber type conversion, and in NMJs, increasing the number of pre- and post-synaptic components.<sup>5,6</sup> Briefly, physical exercise is used as a treatment in postmenopausal women to

improve functional capacity, coordination, muscle strength and quality of life.<sup>7,8</sup> One treatment modality for muscular system changes uses mechanical vibrations. These are provided by vibratory platforms, which are presented as a method of easy applicability and shorter duration compared to other exercise modalities.<sup>9</sup> In muscle tissues, these vibrations, after changing the muscle-tendon length, lead to maximum muscle strength gaining and hypertrophy because it requires contraction and posture maintenance during exercise; as such, this method can be successful in treating changes caused by hormonal deprivation in muscle tissues.<sup>10,11</sup>

However, there are few data in the literature regarding the effects of hormonal deprivation on NMJs and muscle fiber types as well as the possible changes that mechanical vibration may cause in these structures. In addition, this is a search for relevant information about the treatment of disorders arising from hormonal deprivation, a process inherent to women. Thus, the present study aims to histomorphometrically evaluate neuromuscular junctions and the muscle fibers types in the soleus muscles of oophorectomized Wistar rats submitted to a mechanical vibration treatment on a commercial platform for 4 weeks.

## Materials and Methods

This is an experimental, cross-sectional and quantitative study performed at the Laboratory of Injury and Physical Therapeutic Resources Study and in the Laboratory of Structural and Functional Biology of our institution. The research

project was authorized by the Ethics Committee in Animal Use – CEUA Unioeste.

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  $23 \pm 1^\circ\text{C}$  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 8<sup>th</sup> week of life, using the protocol from Khajuria et al,<sup>12</sup> 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 (Paulínia, 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 scalpel blade, was performed to access the peritoneal cavity; adipose tissues were removed until the identification of the uterine tubes and ovaries. After organ recognition, a simple catgut 4.0 suture was performed in the uterine horn area, promoting bilateral ovary resection. At the end of the procedure, internal sutures with 4.0 simple, reabsorbable catgut sutures were performed, while external sutures used 4.0 nylon.

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.<sup>12</sup>

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,<sup>13</sup> 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 8<sup>th</sup> 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.<sup>14</sup> 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,<sup>15</sup> 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 12<sup>th</sup> 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 antimeres were removed and immersed in Karnovsky fixative<sup>16</sup> at 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.<sup>17</sup> 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 antimeres was collected and kept at room temperature for 40 minutes.<sup>18</sup> Next, for tissue preservation, the material was covered with neutral talc according to the technique by Moline et al,<sup>19</sup> frozen in liquid nitrogen for 2 minutes, stored in cryotubes and kept in Biofreezer (Lupetec, São Carlos São Paulo, Brazil) at  $-80^\circ\text{C}$  for histoenzymology analysis. Frozen muscle segments were transferred to a Lupetec CM 2850 Cryostat Microtome cryostat chamber (Lupetec, Vila Monumento, SP, Brazil) at  $-30^\circ\text{C}$  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  $7\mu\text{m}$  in thickness. For analysis of the oxidative and glycolytic metabolism of muscle fibers, sections were submitted to a nicotinamide adenine dinucleotide – tetrazolium reductase (NADH-TR) reaction, according to the Pearse<sup>20</sup> technique modified by Dubowitz et al.<sup>21</sup> This analysis quantifies and measures the percentages of different muscle fibers types (I, IIa 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).

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

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

**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.

## 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).

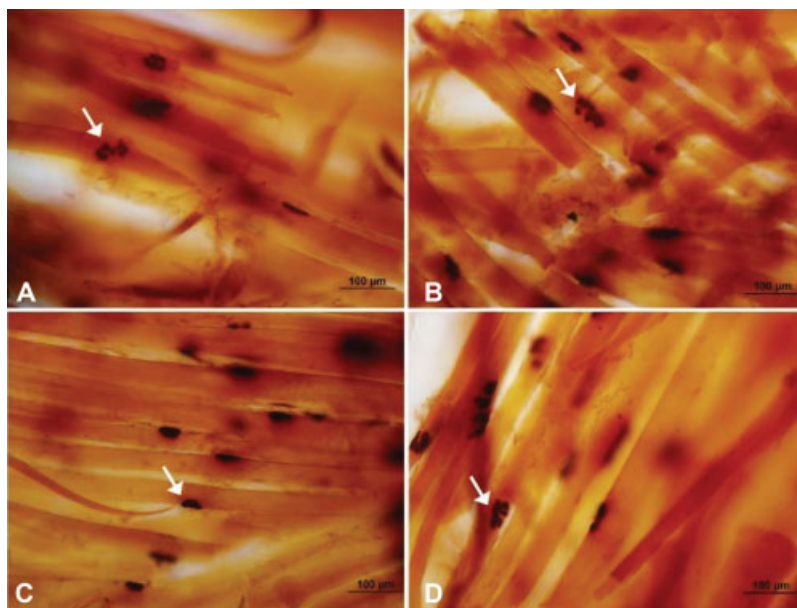
### Type of Muscle Fibers

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.

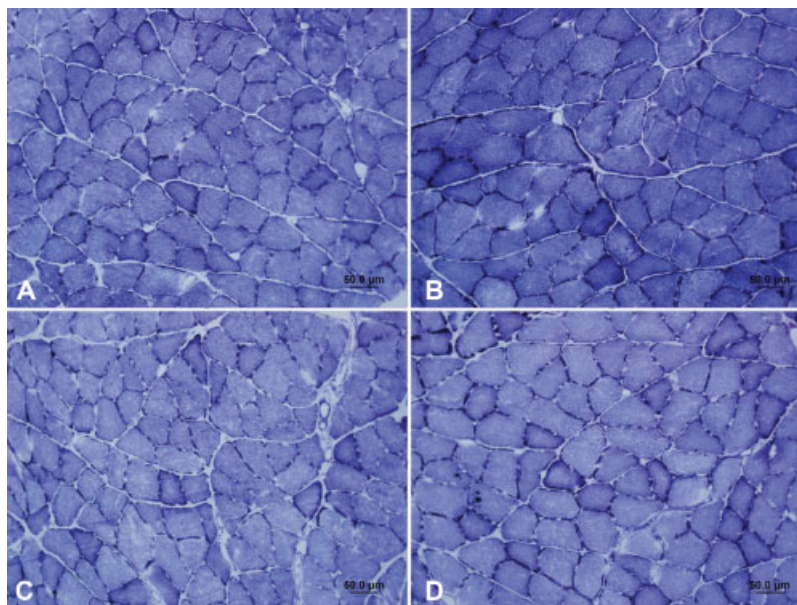
## Discussion

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<sup>4</sup> 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.<sup>22</sup> However, the changes



**Fig. 1** Photomicrographs of neuromuscular junction (NMJ) receptors of the soleus muscle of Wistar rats after nonspecific esterase reaction. A, pseudo-oophorectomy group (GP); B, pseudo-oophorectomy and treatment group (GPT); C, oophorectomy group (GO) with decreased NMJ area and diameter at the morphological comparison with other groups; D, oophorectomy and treatment group (GOT). Arrowhead represents NMJs receptors labeled by this technique.



**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.

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.<sup>23–25</sup> Thus, degeneration of the NMJs may be triggered by disturbances in muscle metabolism followed by changes in motor units.<sup>26,27</sup>

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,<sup>28</sup> which may result in sensitization and loss of motor units, mass decrease and muscle atrophy<sup>29</sup> due to degeneration of the NMJ,<sup>26,27</sup> corroborating our findings.

Deschenes et al<sup>2</sup> 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<sup>30</sup> 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.<sup>1</sup> The resistance training performed by Deschenes et al,<sup>25</sup> 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,<sup>23</sup> 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<sup>1</sup> attempted to evaluate the differences in NMJs from oxidative and glycolytic fiber, 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 sarco-plasmic 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<sup>1,23</sup> 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

literature, but more specific methodologies are required for its confirmation.

Haizlip et al<sup>4</sup> 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, leading to adaptation, improved transmission, and hypertrophy of the NMJs.<sup>3,23,31</sup>

The soleus muscles from all groups were mainly composed of type I fibers, which have the highest mitochondrial respiration capacity,<sup>3</sup> and the NADH-TR technique was used to determine the oxidative functioning of the mitochondria by an enzymatic reaction staining.<sup>32</sup> The oophorectomy did not affect the percentages of each fiber type in murine soleus muscles in a study by Moran et al,<sup>33</sup> but the immunohistochemical analysis revealed different types of fibers. Haizlip et al<sup>4</sup> 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.<sup>1</sup> Camargo Filho et al<sup>34</sup> 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-TH reaction mostly identified oxidative fibers. Deschenes et al,<sup>2</sup> 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 IIa), unlike more superficial muscles, such as the gastrocnemius (fibers IIx and IIb).<sup>4</sup> Since the present study used the soleus muscle, which is main postural and oxidative muscle in rats,<sup>3</sup> 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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