Alterations in neuromuscular junctions and oxidative stress of the soleus muscle of obese Wistar rats caused by vibratory platform training

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Abstract

Objectives: evaluate the effects that whole-body vibration (WBV) causes on the neuromuscular junctions and oxidative stress of the soleus muscle of obese Wistar rats. Methods: 32 male Wistar rats were used, 16 of which were obesity induced by monosodium glutamate, randomized into four groups: control (GC), control with WBV (GCP), obese (GO) and obese with WBV (GOP). At the 70 days old, the training on WBV was started, performed 3 times a week, during 8 consecutive weeks. At the 130 days old, the animals were euthanized and the soleus muscles were collected. Results: Regarding the analysis of the neuromuscular junctions, the obese groups had lower mean size when compared to the control groups. On the other hand, the WBV presented higher averages when compared to the groups that did not perform the training. Regarding the oxidative stress, for the lipid peroxidation there was a significant difference between obese and non-obese animals, however, there was no difference between the animals WBV and those who did not. Conclusion: WBV promotes beneficial changes such as increased measurements of the structures of the neuromuscular junctions, but is not able to promote changes in the concentration of the cholinesterase enzyme in the synaptic cleft.

Keywords: Neuromuscular Junction, Obesity, Oxidative Stress, Soleus Muscle, Vibrations

Introduction

Obesity is a global disease that involves several psychological, social and economic consequences, in addition to cause a decline in the quality of life of individuals. The accumulation of adipose tissue promotes a complex range of physiological and pathological alterations in the body, including increased amounts of inflammatory cytokines such as TNF-α, IL-1, IL-6 and C-reactive protein that are harmful to skeletal muscle, promoting degradation and reduction of muscle protein synthesis.

Among the structures that make up the musculoskeletal tissue is the neuromuscular junction (NMJ), which represents the interaction between the peripheral nervous system and the muscular tissue, comprising the presynaptic axon, synaptic cleft, muscle fiber, vesicles, acetylcholine receptors and also by cholinesterase enzyme, which is the key enzyme of nerve impulse transmission and responsible for terminating neurotransmission by hydrolysis, cleaving acetylcholine into acetic acid and choline.

Any morphological change in the NMJ, and/or communication on the motor terminal plate, can result in impaired communication between the peripheral nervous system and the musculoskeletal system, preventing the...
correct action of excitation-contraction, causing decline in muscle contractility and failure in neuromuscular transmission\(^6\).

However, the NMJ has a high plasticity and the physical exercise is able to reverse changes, promoting increase in the amount, length and complexity of branches, in the synaptic cleft perimeter and in the end plate dispersion\(^6\).

In the condition of obesity, it is common to find an increase in the production of inflammatory substances, in addition to an increase in the lipoperoxidation reaction (LPO), which induces overproduction of reactive oxygen species (ROS). In addition, it is also common in obesity, the antioxidant defense activity is reduced, expressed by the decrease in the enzymes superoxide dismutase (SOD) and catalase (CAT), among others. This imbalance characterizes the so-called oxidative stress\(^7\).

Lipoperoxidation is one of the principal markers of oxidative stress. ROS, mainly superoxide and hydroxyl, have a preference for the oxidation of fatty acids, yet, this enzymatic activity is involved in damage and changes in the content of DNA and cell structures, with the damage of biological membranes, allowing intracellular calcium to enter, mitochondrial edema, lysis and cell death\(^6\), resulting in the development of associated diseases\(^8\).

In the antioxidant system, SOD, which is an intracellular enzyme and is present in oxygen metabolizing cells, decomposes the superoxide radical into hydrogen peroxide, which has a less toxic potential. Hydrogen peroxide is generated by incomplete reduction of oxygen in electron transport systems or as enzymatic by-products, forming several other ROS. Following the complex cascade of the antioxidant system, CAT, in turn, promotes the neutralization of hydrogen peroxide in more stable molecules, such as water and oxygen\(^9\). Thus, due to this insufficient antioxidant system, obese individuals are more susceptible to oxidative damage\(^10\), condition that contributes to the development of various human diseases such as cancer and neurodegenerative diseases\(^11\).

However, markers of oxidative and inflammatory damage, besides being influenced by age and body composition, also change according to physical activity\(^12\). Sedentarism and physical inactivity lead to muscle disuse and cause increased production of oxidative enzymes\(^13\). However, physical exercise, through chronic contractile activity, also continuously produces ROS\(^14-16\), but regular physical exercise in obese individuals decreases the levels of systemic oxidative stress and in skeletal muscle from three months of training against resistance\(^12\).

The use of whole-body vibrations (WBV) is a modality of physical exercise that has been extensively used to promote improvements in muscle tissue. Vibrations generated by the vibrating platform produce periodic changes in the acceleration and displacement of body mass\(^17\). With this, the changes caused by this type of training can be explained by Newton's Second Law, which says that the force (F) of an object is equal to the mass (M) multiplied by its acceleration (A) (F= M\(\times\)A)\(^18\). Thus, vibrations induce muscle hypertrophy, improve strength and potency, being considered an alternative method to combat muscle changes caused by obesity\(^18,19\).

In the literature it is described that obesity negatively influences muscle tissue. The state of obesity promotes a decrease in the cross-sectional area, in the number of nuclei and capillaries per fiber, in addition to inducing an increase in connective tissue. Just as it is known that exercise using vibrations is able to increase the number of nuclei and capillaries per fiber and decrease the amount of connective tissue induced by obesity\(^20\). However, it is still unclear whether the changes in the soleus muscle, caused by the WBV exercise, reflect changes in the NMJ and in the oxidative and antioxidant system, especially in relation to animal models of obesity. Thus, the present study was designed to verify the alterations in the neuromuscular junctions and in the oxidative stress of the soleus muscle of obese Wistar rats caused by training on a vibratory platform.

**Methods**

The present study had an experimental, transversal and quantitative character, developed in the Universidade Estadual do Oeste do Paraná (UNIOESTE). The research project was approved by the Ethics Committee for Animal Use (CEUA) of UNIOESTE.

**Experimental groups**

For the present experimental model, 32 male Wistar rats were used, of which 16 were obese (with obesity induced by monosodium glutamate [MSG]), that were equally randomized into four groups: Control Group (CG - sedentary control/without intervention, n=8), Control Group + Vibratory Platform (GCP – vibratory platform control, n=8), Obese Group (GO – MSG sedentary/without intervention, n=8), Obese Group + Vibratory Platform (GOP – MSG + vibratory platform, n=8). The animals were kept in standard polypropylene boxes, in an environment with a temperature of 22±1°C, with a photoperiod of 12 hours, receiving water and feed ad libitum.

**Obesity induction protocol**

The protocol for the induction of obesity was performed during the first five days of life of the animals, with the application of an intradermal injection per day of MSG, at a dose of 4 mg/g body weight, the remaining animals were administered hyperosmotic saline solution, similar to a daily dose of 1.25 mg/g body weight\(^21\).

**Training protocol**

At 70 days of age, the intervention was started and the commercial Vibro Oscillatory platform of the brand Arkutus® triplanar professional (Santa Tereza do Oeste, Paraná, Brazil) was used, three times a week, lasting 10 minutes/day, for eight consecutive weeks. The protocol used was adapted...
to a frequency of 60 Hz, with a vibration amplitude of two millimeters\(^2\). To position and contain the animals on the vibrating platform, an MDF wooden support was used, with stalls, made according to the dimensions of the device, so that it did not keep contact with the base of the platform and thus did not interfere with the vibrations. In each training session, the animals were rotated in their positioning in the stalls to minimize biases resulting from the difference in vibration amplitude in different areas of the platform.

**Euthanasia**

At 130 days of age, after the end of the training period, all animals were euthanized by anesthetic overdose, with application of xylazine 30 mg/kg and ketamine 300 mg/kg (Ceva Brasil\(^{®}\)) intraperitoneally, and the left pelvic limb soleus muscle was then collected. The muscle tissue was dissected, removed, weighed and sectioned into fragments using a stainless-steel blade.

**Histological processing of soleus muscle and analysis of neuromuscular junctions**

For the study of NMJ, the proximal fragments were immersed in Karnovsky solution at room temperature. To assemble the slides, the muscles were sectioned longitudinally, in small portions, with the aid of stainless-steel blades. After performing the sections, they were submitted to unspecified Sterase reaction. Subsequently, the slides were made and the morphometric parameters were measured, analyzed using a light microscope, the visual fields were photomicrographed at 200x magnification and the area, largest and smallest diameter of 150 JNMs per animal were measured, and analyzed with the aid of the Image-Pro-Plus 6.0 program (Media Cybernetics, Maryland, USA).

**Preparation of biological material**

For enzymatic analyzes the distal fragments were removed and immediately homogenized in 1 milliliter (mL) of Tris HCl (Sigma\(^{®}\)) buffer pH 7.4 and centrifuged at 12,000 rotations per minute (rpm) for 10 minutes at 4°C. The homogenate was frozen at -80°C for subsequent protein dosage. The protein quantification of the samples was determined by the Bradford method, using bovine serum albumin as standard. So, all samples were normalized to 1 milligram (mg) of protein/mL.

**Cholinesterase enzyme analysis**

The enzymatic dosage was performed, associated with neurotoxicity by the activity of the cholinesterase enzyme (ChE), analysis by the Ellman method, modified, whose principle is the measurement of the production of thiocoline when acetylthiocholine is hydrolyzed. This is done by the continuous reaction of thiol with 5,5-dithiobis-2-nitrobenzoate ion to produce the yellowish stained anion of 5-thio-2-nitrobenzoic acid. The reaction was performed in triplicate in 300 μL of solution, containing 0.05 mM of 5,5-Dithiobis-2-nitrobenzoic acid (Sigma\(^{®}\)) (DTNB) and 1.5 mM of acetylthiocholine (Sigma\(^{®}\)) (ATC).

ChE activity was calculated in relation to the protein concentration (mg/mL) using the molar extinction coefficient of the DTNB (1.36mM-1.cm-1). The protein quantification was calculated from the raw sample and the reading was performed at 22°C. The results were expressed in nmol. min1.mg protein-1.

**Oxidative stress analysis**

The determination of lipoperoxidation reaction (LPO) was performed in order to indirectly quantify the peroxides, thus reflecting the intensity of lipid peroxidation. Thiobarbituric acid (TBARS) method was performed by comparing absorbance with Malondialdehyde standard curve (MDA), the main by-product of cellular lipid peroxidation. For the sample preparation, the middle, containing an aliquot of 0.33 mg/mL of protein in the sample in trichloroacetic acid (Sigma-Aldrich\(^{®}\)) (TCA) 6.7% in final volume of 180 μL, was agitated in vortex, left in an ice bath for 5 minutes and centrifuged for 5 minutes at 12000 rpm at 4°C. For the dosage of reactive substances to TBARS 40 μL of the supernatant, as well as of different concentrations of MDA (Aldrich\(^{®}\)) were added in microplate, in triplicate, in reaction medium, containing 21.42 mM of TBARS (Sigma-Aldrich\(^{®}\)), 17.86 mM of NaOH (Labsynth\(^{®}\)) (used for TBARS solubilization), 0.73 M of TCA (Sigma-Aldrich\(^{®}\)), 0.032 mM of BHT (Sigma-Aldrich\(^{®}\)), 3% ethanol (Neo\(^{®}\)) (used for BHT solubilization) in PBS. The reaction was read at 22°C; after 60 minutes of incubation, at 60°C, at an absorbance of 535 nm. The results of lipid peroxidation were expressed in nmol of MDA.mg of protein-1.

**Analysis of the antioxidant system**

For the activity of superoxide dismutase (SOD), the modified Crouch’s method was used. The principle of this analysis is to quantify the complex formed between superoxide and tetrazolium blue (NBT), measured at 560nm for 1.5 hours. An aliquot of 0.75 mg/mL of protein in 25% ethanol (Neo\(^{®}\)) was prepared in a volume of 800 μL and centrifuged at 12000 rpm (4°C) for 20 minutes. From the supernatant, the middle of the reaction was prepared in a 96 well microplate. In triplicate, in final volume of 200 μL, containing 0.1mg of protein/mL-1, 0.09 mM of NBT (Sigma-Aldrich\(^{®}\)), 0.015 mM of EDTA (Neo\(^{®}\)), 34.78 mM of hydroxylamine sulphate (Sigma-Aldrich\(^{®}\)), 79 mM of sodium carbonate buffer (Sigma-Aldrich\(^{®}\)) pH 10.2 and the plate read at 22°C. One unit of SOD in nmol. min-1.mg of protein-1.

Activity of Catalase (CAT) was accompanied by a decrease in absorbance at 240nm, from the principle of peroxide dismutation, whose molar extinction coefficient is 40 M-1. cm-1. The duplicates, in 2 mL of solution, in a quartz cuvette, presented a final concentration of 0.01mg of protein.mL-1, and the middle of reaction presented final concentrations of 13.5 mM of H2O2 (Sigma\(^{®}\)), 50 mM of TRIS-HCl (Sigma\(^{®}\))
pH 8.0 and 0.25 mM of EDTA (Neon®). The results of the catalase enzyme activity were expressed in mmol.min-1.mg of protein-1.

**Statistical analysis**

The data obtained were tabulated in Excel 2010 sheets and were expressed and analyzed by means of descriptive and inferential statistics, were evaluated for the presence of outliers through the diagnosis of exclusion of regression, whose values were replaced by the mean of the group. They were then analyzed for normality using the Shapiro-Wilk test and for homogeneity using the Bartlett’s test. The results were evaluated as to their significance by means of the Analysis of Variance (Anova) of two factors, considering the obesity factor, with the obese and non-obese levels and the exercise factor, with the exercise and non-exercise levels.
The follow-up test used was the Tukey-HSD test. The level of significance adopted was 5%, and the R 3.5.1 program was used for all tests (R Core Team, 2018).

Results

Regarding the analysis of the neuromuscular junctions (Figure 1 A-D) it can be observed that in the area of section there was significant difference between the groups (OBESITY - F=291.048; p<0.01; PLATFORM - F=10.225; p<0.01). The obese groups, GO and GOP, had lower mean cross-sectional area when compared to the control groups (GC and GCP), demonstrating changes caused by obesity. On the other hand, the GCP and GOP groups submitted to exercise on a vibratory platform presented higher averages when compared to the groups that did not perform the training (GC and GO), indicating that the exercise is capable of increasing the size of the area of section of the JNM. However, there was no significant interaction between the factors “OBESITY” and “PLATFORM” (F=3.381; p=0.07) (Figure 1E).

Regarding the larger diameter of the neuromuscular junctions, there was also a significant difference between the groups (OBESITY - F=257.518; p<0.01; PLATFORM - F=37.990; p<0.01). The obese groups, GO and GOP presented smaller mean diameters when compared to the control groups (GC and GCP), and also, the groups submitted to vibratory platform exercise (GCP and GOP) presented higher averages when compared to the groups that did not perform the training, GC and GO, indicating that the training was able to increase this diameter. However, there was no significant interaction between the factors “OBESITY” and “PLATFORM” (F=1.337; p=0.25) (Figure 1G).

Regarding the activity of the ChE enzyme, there was no significant interaction between the groups (OBESITY - F=0.31, p=0.57; PLATFORM - F=0.80, p=0.37) and between the factors (F=2.44, p = 0.12) (Figure 2).

Regarding the oxidative stress, there was no difference
between the groups for this enzymatic activity and for lipid peroxidation, there was a significant difference between obese and non-obese animals (F=4.67, p=0.03), with a higher rate of lipoperoxidation in these animals. However, there was no difference between the animals who underwent training on the vibratory platform and those who did not (F=1.10, p=0.29) and between the factors “OBESITY” and “PLATFORM” (F=0.06, p=0.80). In the antioxidant system, for the SOD enzyme there was no significant difference between the groups (OBESITY - F=0.53, p=0.47; PLATFORM - F=2.29, p=0.13), and there was no interaction between the factors “OBESITY” and “PLATFORM” (F=1.81; p=0.18). For the catalase enzyme there was also no significant difference between the groups (OBESITY - F=0.68, p=0.41; PLATFORM - F=0.68, p=0.41), and there was no interaction between the factors “OBESITY” and “PLATFORM” (F=0.45; p=0.50) (Figure 2).

**Discussion**

The vibration stimulus induces changes in the musculotendinous length by stimulating the receptors sensitive to the stretch reflex, thus activating the muscle spindles. Vibrations increase the firing of the afferent fusel fibers by means of type Ia fibers, excite the alpha motoneurons, thus having the proprioceptive activation of the sensory system, resulting in muscle contraction, through the tonic vibration reflex (RTV). However, they also issue commands to the type Ib afferent inhibitory fibers of the Golgi tendon organs (OTG) that are sensitive to variations in tension, thus having synchronous contractions of the concentric and eccentric muscles.

The neuromuscular junction (NMJ) is also responsible for the contractile activity of the skeletal muscle, having the function of transmitting nerve impulses between motor neurons and muscle fibers. The NMJ has high plasticity, however little is known about the results of exercises with the use of vibrations in this structure.

Neuromuscular junctions may suffer changes in quantity and size in several situations, such as psychiatric disorders, metabolic diseases, aging and genetic diseases, as well as muscle disuse induced by physical inactivity is also capable of significantly reducing the dimensions of the area and the length of neuromuscular junctions; however, exercise is important to reverse these changes.

As a result, it was possible to observe that both obesity and the use of the WBV were able to promote changes in the size of the neuromuscular junctions, especially in the larger diameter issue. However, there are no specific studies in the literature on the action of obesity in the neuromuscular junction, as well as no research to demonstrate the changes caused by mechanical vibrations in this structure.

In order to determine the effects of exercise on NMJs, Deschenes et al. evaluated the influence of endurance training on the morphology of NMJ of the musculature of young and elderly rats. The animals that were trained on a treadmill for a period of 10 weeks, five days in the week with a duration of 60 minutes of exercise, presented after the intervention exercise-induced adaptations, such as increase in the amount and length of the terminal branches of NMJs, but this finding was found only in younger rats. The results of the present study are in line with what was ascertained in this research, since the animals studied were also young, at approximately 20 weeks of ages, who have a high capacity to adapt to metabolic stimuli. Krause Neto et al. in a systematic review found different exercise protocols, with different intensities and duration, and concluded that regardless of age, type and frequency of exercise; physical activity is able to modify the components of NMJ throughout life.

Seiene, Umnova and Kaasik investigated the differences in JNMs in different types of young animal fibers after six weeks of resistance exercise training. The authors found that the endurance training, it increased the axon terminal area in both types of fibers, glycolytic and oxidative, but this result is more evident in oxidative fibers, which have a faster rate of muscle protein renewal, in addition to a higher number of mitochondria and also a higher number of vesicles for acetylcholine resynthesis after synapse. It should be noted that in the present study a muscle composed basically of oxidative fibers was used, so the type of fiber would not influence the analysis of the results.

Also present in the synaptic cleft, the enzyme cholinesterase (ChE) has the function of removing acetylcholine (ACh) quickly, avoiding the repeated activation of acetylcholine receptors (AChR) in response to a single action potential in the nerve. The ChE hydrolyzes ACh released from the motor’s nervous terminal, and thus ends its action. This process is fast and very important, because this way, after the release of ACh, AChR are activated only once, preventing multiple openings of the channels regulated by AChR, due to the persistent presence of ACh in the synaptic cleft.

As for the activity of cholinesterase in this study, no significant differences were observed between the groups, demonstrating that the intervention was able to modify the amount of neuromuscular junctions without, however, producing changes in the amount of ChE in the synaptic cleft. The impairment of ChE activity would imply an increase in the time of ACh available in the synaptic cleft, causing sustained activation of AChR and resulting in excessive calcium influx through the channels regulated by AChR, thus, emphasize that the training on vibrating platform did not promote neurotoxicity, for excess of acetylcholine in the musculoskeletal tissue of rats in this obesity model.

Consequently, the impairment of ChE activity implies an increase in ACh time in the synaptic cleft. The sustained activation of AChR, due to ChE deficiency, causes an excessive influx of calcium through the channels regulated by AChR and results in the activation of intracellular proteases, which damage the postsynaptic membrane. Therefore, proper AChE activity is essential for maintaining normal NMJ.

In addition to the fact that the deficiency in ChE results in the activation of intracellular proteases, which damage the postsynaptic membrane, thus it is concluded that the
proper activity of AChE is essential for the maintenance of normal NMJ.

In a study that analyzed the activity of ChE in different tissues and different ages, Strauss et al.\textsuperscript{36} observed that in older animals aged 11 to 16 weeks there is a decrease in enzyme activity in the gastrocnemius muscle, thus, the detection of changes in the action of this enzyme becomes difficult due to its low activity in this tissue, thus correlating these results to those found in this study, in which the animals on the date of euthanasia had more than 16 weeks, but other more specific techniques could detect minimal changes in cholinesterase with greater precision.

With regard to the activity of oxidative enzymes, there was a significant difference only in lipid peroxidation, with a decrease in the activity of LPO in obese animals when compared to non-obese animals, demonstrating that oxidative stress is minimized in obese animals. In a study with obese animals, by hyperlipidic diet, underwent treadmill training, there was a decrease in LPO and also in SOD\textsuperscript{36}, and the reduction in the latter differs from the findings of the present study. It is considered that this occurrence may have been caused by the difference in exercise duration time and weekly frequency, since it was performed for 60 minutes and five days a week characterizing an exercise with greater intensity than that performed by the animals in this study, demonstrating that more intense exercises, which induce exhaustion of the musculoskeletal system, initially culminate in increased oxidative stress due to the generation of EROS, which exceeds the capacity of defense in skeletal muscle\textsuperscript{37}, a fact that was not observed by training on vibration platform, that is, it is believed that the duration of 8 weeks of protocol was sufficient for the antioxidant system to enter into balance.

On the other hand, a study carried out with obese men submitted to 12 weeks of cycloergometer training, three sessions per week lasting 60 minutes, despite observing that neither obesity nor resistance training altered the protein content of CAT, found that the concentration of LPO in obese individuals was higher than in thin individuals, and after being submitted to physical exercise there was a decrease in lipoperoxidation in obese individuals, in addition, also showed increased activity of SOD\textsuperscript{36}. This difference is attributed to the hypothesis that animal models of obesity induction promote changes in fatty acid metabolism reflecting results contrary to those found in studies with humans\textsuperscript{38}. Thus, it is understood that other future studies with different animal models and different muscle groups could find results that differs from the present study, being necessary to confront these controversial data.

There are few studies in the literature that have analyzed the effects of the use of the vibratory platform on the activity of oxidative enzymes, in a study that investigated the effects of body vibration exercise on lipid metabolism and oxidative stress in diabetic and obese animals, submitted to training on the vibratory platform at a frequency of 45 Hz, 60 minutes/day for 12 weeks. It also found no differences in the enzymatic activity of CAT and SOD, but exercise was able to reduce the LPO\textsuperscript{39}, results similar to those of the present study, demonstrating that even with differences in the duration and intensity of exercise, the platform did not promote oxidative stress that is induced by physical exercise.

Some authors have demonstrated that physical exercise promotes an imbalance between the oxidant and antioxidant systems\textsuperscript{40–42}. Thus, it can be seen that the exercise proposed through the use of body vibrations did not promote oxidative stress in the soleus muscle, since it did not alter the activity of the enzymes CAT and SOD, demonstrating that there is a perfect balance between the antioxidant enzymes for the maintenance of cellular integrity even when training on vibration platform.

**Conclusion**

Vibratory platform training promotes beneficial changes such as increased measurements of the structures of the neuromuscular junctions; but is not able to promote changes in the concentration of the cholinesterase enzyme in the synaptic cleft. In addition, the proposed exercise did not promote oxidative stress in muscle tissue, maintaining the balance between the enzymes studied. It was also noted that the obesity induction model used in this study promotes a decrease in muscle lipoperoxidation in Wistar rats.

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