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Physical Exercises during Youth Regardless of Nandrolone Decanoate Use Prevent Neuromuscular Morphological Alterations Caused by Aging

*Erick Valentino[1,2#], Ana Paula Silveira Leite[1,2], Carina Guidi Pinto[1,2], Felipe Cantore Tibúrcio[1,2], Paula Aiello Tomé de Souza Castro[3] and Selma Maria Michelin Matheus[2]**

[1]São Paulo State University (Unesp), Medical School, Botucatu, São Paulo, Brazil.

[2]Department of Structural and Functional Biology (Anatomy Sector), São Paulo State University (Unesp), Institute of Biosciences, Botucatu, São Paulo, Brazil.

[3]Department of Physical Therapy, Center for Biological and Health Sciences, Federal University of São Carlos (UFSCar), São Carlos, São Paulo, Brazil.

[#]In memoriam

Abstract

Skeletal muscle aging is characterized by loss of muscle mass and function caused by a reduction systemic hormone. Physical exercises have beneficial effects in lean mass and may influence the Neuromuscular Junctions (NMJs). Anabolic androgenic steroids, such as Nandrolone Decanoate (ND), are widely used among athletes, but can cause a decrease of testosterone. The objective of this study was to evaluate the impacts of a supraphysiological dose of ND with or without physical exercises during youth on soleus muscle and their NMJs. Twenty 90-day-old male Sprague-Dawley rats were treated for 8 weeks and distributed into sedentary or exercised groups, with or without ND use (twice/week, 5 mg/kg, im.). Physical exercise was conducted by jumping in water three times per week. At the age of 300 days, muscles were collected, and the analyses were performed: Morphological, morphometric, and ultrastructural analysis of muscle fibers and their NMJs; immunohistochemistry and morphometric analysis of fast- and slow- muscle fibers; and confocal microscopy for Acetylcholine Receptors (nAChRs). Results demonstrated that there was a weight decrease in the animals but not in the muscle that got ND. The morphology and morphometry of the NMJs remained steady, and regarding ultrastructure, the junctional folds were scarce. The animals that exercised had a pattern of nAChRs in continuous branches, and in sedentary groups, the "island" pattern was present. The pattern of slow- and fast-twitch muscle fibers remained stable in all groups. Central nuclei and focal areas of injury as well as myofibrillar disorganization were observed in the animals that got ND. Thus, the alterations observed in this study were consequences of the aging process and physical exercises performed in youth maintained the structural pattern of nAChRs that characterizes young animals. ND did not prevent morphological changes in the neuromuscular systems that are consequences of aging.

Keywords: Aging; Nandrolone decanoate; Physical exercise; Neuromuscular junction

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Correspondence:
Selma Maria Michelin Matheus, Department of Structural and Functional Biology (Anatomy Sector), Institute of Biosciences, São Paulo State University, 250, Botucatu, São Paulo, Brazil,
E-mail: selma.matheus@unesp.br

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Introduction

Physical exercise has been regarded as an important factor in the adaptation process of skeletal muscle [1, 2]. Systematic physical training can cause modulation of muscle fibers in response to metabolic overcompensation to meet the need of repeated body stimuli and optimize physical performance [2].

Muscle activity may influence both pre- and post-synaptic elements of the Neuromuscular Junctions (NMJs) over time. Voluntary exercises started in middle age can inhibit the loss of nerve terminals that occurs in elderly NMJ [3].

Aging is a factor that has been proven to affect the neuromuscular system [4,5]. Skeletal muscle age is characterized by progressive loss of muscle mass and decreased muscular function [6,7]. This deficit associated with aging is known as sarcopenia, which intensely affects elderly life's quality and predisposes them to morbidity, mortality and disability increased risk [6,7].

Sarcopenia is multifactorial and involves both intrinsic and extrinsic factors. However,

several studies conducted in animals and humans suggest that the degeneration of motor neurons, followed by alterations in the structural and functional NMJ integrity, functional denervation and loss of motor units that's contribute to the significant progression of the skeletal muscle aging [5].

The muscle metabolic capacity can be further affected by changes in the levels of systemic hormones [7]. Testosterone and its synthetic derivatives can increase lean body mass, muscle strength and synthesis of muscle protein [8]. Although several hormones are known as effectors of skeletal muscle metabolism, testosterone is one of them that have been extensively studied [9].

Testosterone acts by increasing the number of progenitor muscle cells and promoting their myogenic differentiation [10]. It also stimulates mitochondrial biogenesis which increases the oxygen supply to tissues, the red cell number, and capillarity [11].

Nandrolonedecanoate (ND) is a synthetic testosterone derivative (Ganesan et al., 2020). It is the most commonly used anabolic androgenic steroid (AAS) among athletes, especially males, since it has few adverse effects (Riezzo et al., 2014). On the other hand, it is described in literature that anabolic steroids associated with endurance exercises induce a reduction in total and free testosterone levels (Shokri et al., 2010), which can cause a loss of muscle mass and strength (Hanson et al., 2020).

Considering the anabolic steroids abuse and the common use among athletes the objective of this study was to evaluate the impacts of a supraphysiological dose of ND with or without physical exercise during youth on the skeletal muscle aging process.

Material and Methods

Animals and experimental design

Twenty 90-day-old male rats Sprague-Dawley obtained from the Multidisciplinary Center for Biological Investigation (CEMIB) of the State University of Campinas (Unicamp), São Paulo, Brazil were used. These animals were kept during the trial period in the animal research laboratory of the Department of Structural and Functional Biology (Anatomy Sector), Institute of Biosciences (IBB), São Paulo State University (Unesp), Botucatu, São Paulo, Brazil, under appropriate conditions and approved by the Ethics Committee in the Use of Animals (CEUA - IBB/Unesp), protocol number: 448.

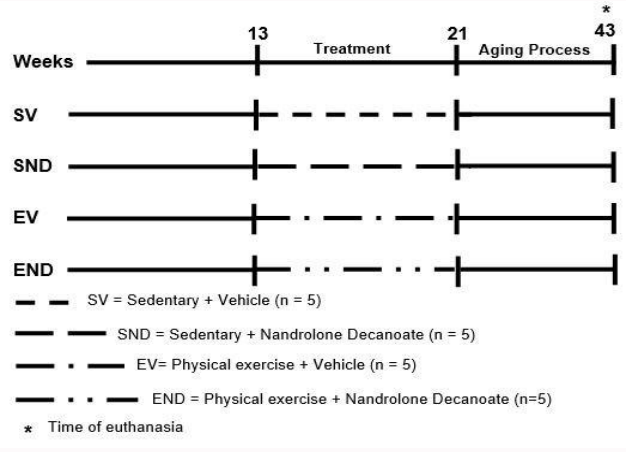

Figure 1: Experimental design in which the animals are represented according to their groups.

The animals were divided into four experimental groups and were treated for 8 weeks and distributed according to the experimental design (Figure 1).

Experimental protocol

The ND groups, SND (Sedentary + ND) and END (Physical exercise + ND), received Intramuscular (IM) injections of Deca-Durabolin® (Schering-Plough, São Paulo, Brazil) containing 10 mg/kg/week (5 mg/kg body weight twice a week) according to the protocol developed by Shokriet al. [12], for 8 weeks. The dosage used was 10 to 100 times more than the therapeutic dose and was considered a supraphysiological dose [13]. The SV (Sedentary + vehicle) and EV (Physical exercise + vehicle) groups received injections (IM) containing only the vehicle, propylene glycol (0.2 mL/kg body weight) applied following the same procedure.

Resistance physical training: Jumping in a liquid medium

The animals from EV and END groups were subjected to a training program of jumping sessions in a PVC cylinder containing 30°C water at a depth of 38 cm [14]. In the first five days prior to the 1st injection, the animals from the exercise groups had an adaptation period before exercising in a liquid medium. During this period, they initially performed 2 series of 5 jumps that were gradually increased until they reached 4 series of 10 jumps. An overload weight vest was placed on the anterior area of the animal's chest. Physical training lasted 8 consecutive weeks. The training program of jumps in a liquid medium with an overload weight was conducted three days a week. The session consisted of four series of ten jumps each with a progressive increase in weight: 50% of body weight (second and third weeks), 60% (fourth and fifth weeks) and 70% (sixth, seventh and eighth weeks) [15]. The animals were removed from the water during the rest period of 60 seconds between the series.

Throughout the whole physical training period, the sedentary animals (SV and SND) did not undergo the jump sessions and were placed in a box with shallow water, also at 30°C, to have contact with water without needing to jump.

The animals were dried with a cotton towel and kept at a warm temperature for 30 m after each training session. After being subjected to this experimental protocol, the animals were kept under the regular conditions of the animal research laboratory according to the experimental design until they reached 300 days of age (43 weeks) to characterize aging.

Material processing

After the training and aging period, all animals at 300 days of age were weighed and euthanized. The soleus muscles were then dissected, removed, weighed and processed according to the following protocols.

Morphological and morphometric analysis of NMJs

The soleus muscle middle third (motor point) was fixed in Karnovsky solution and sectioned longitudinally with a razor, the NMJs were dyed using non-specific esterase reaction [16], and had their morphology analyzed. Fifty NMJs of each animal were used to determine the maximum diameter using ImageJ software [17].

Ultrastructural analysis of muscle fibers and their NMJs

Some muscle portions were reduced into fragments, fixed by glutaraldehyde 2.5% and processed according to the Transmission Electron Microscopy (TEM) routine to Electron Microscopy Center of the IBB/Unesp, Botucatu. To identify the muscle fibers and NMJs,

the fragments were imbedded longitudinally, and then the ultra-thin sections were prepared and photographed using TEM Philips (FEI CM100 model).

Laser scanning confocal microscopy analysis

The acetylcholine receptors (nAChRs) of the motor endplate were labeled with rhodamine-conjugated alpha-bungarotoxin (Rh-BTX, Molecular Probes T1175, 1:100 in PBS). The slides were prepared for morphological analysis and photo documentation using the Laser Scanning Confocal Microscope (Leica TCS-SPE), belonging to the Electron Microscopy Center of the IBB/Unesp, Botucatu.

Morphological and morphometric analysis of muscle fibers

The soleus muscle fragments were frozen in liquid nitrogen, slices of 8 µm thick were made (Leica CM 1800 cryostat), and three slides were obtained. The first slide was stained with Hematoxylin and Eosin (HE) and photographed using an Olympus BX41 image analyzer (SC30 camera). The images (200X) were used for general morphological analysis of the muscle and for counting the fibers that had central and peripheral nuclei. For this analysis, approximately 200 muscle fibers selected from 3 to 4 random fields were used. This quantification was obtained using ImageJ software [17].

Immunohistochemistry of the types of muscle fibers (fast and slow twitch fibers)

The other two sides were subjected to immunohistochemistry to identify fast and slow-twitch fibers. The immunoperoxidase method StreptABComplex/HRP was carried out using primary commercial antibodies specific for each protein of the study, including Fast anti-myosin (WB-MYHCf Novocastra 1:160) and Slow anti-myosin (WB-MYHCs Novocastra 1:120).

After identifying the types of muscle fibers (in cross-sections), 5 to 6 fields were photographed per slide to obtain approximately 200 fibers. The fiber types were counted and the area was measured by ImageJ software [17].

Statistical analysis

The results were expressed as mean and standard deviation, and statistically significant differences were considered when $p<0.05$. For the analysis of animal's and soleus muscle weight, maximum diameter of the NMJs, quantification of central and peripheral nuclei and fast and slow fibers areas analysis of variance for the model with one factor were applied complemented by Tukey's multiple comparison test [18]. For analysis of slow and fast fibers numerical quantification it was used two-factor analysis of variance, complemented by Dunn's multiple comparison tests [18].

Table 1: Means and standard deviation of body weight (g) according to group and steroid use; S, sedentary; E, exercise; No, no nandrolone decanoate; Yes, with nandrolone decanoate.

Nandrolone Decanoate				
Variable	Group	No	Yes	p Value
Body	S	539.00(24.08)	485(34.10)	p<0.05
weight	E	498.00(38.83)	443(30.94)	p<0.05
p Value		p>0.05	p>0.05	

Statistical results obtained by scheme-factor analysis of variance and complemented with Tukey's multiple comparison test [18]

Table 2: Means and standard deviation of weight of the soleus muscles (g) according to group and steroid use; S, sedentary; E, exercise; No, nandrolone decanoate; Yes, with nandrolone decanoate.

Nandrolone Decanoate				
Variable	Group	No	Yes	p Value
Soleus	S	0.249(0.011)	0.232(0.023)	p>0.05
weight	E	0.246(0.035)	0.219(0.022)	p>0.05
p Value		p>0.05	p>0.05	

Statistical results obtained by scheme-factor analysis of variance and complemented with Tukey's multiple comparison test [18]

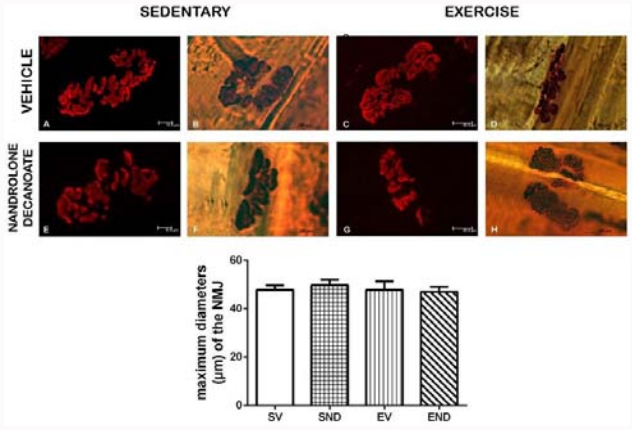

Figure 2: A, C, E and G: Confocal microscopy of the acetylcholine receptors of the soleus muscle identified by rhodamine-conjugated alpha-bungarotoxin. B, D, F and H: Light microscopy findings following the non-specific esterase reaction (Full preparation). I: Means and standard deviation of the maximum diameters (µm) of NMJ of soleus muscles. Statistical results obtained by two-factor analysis of variance, complemented by Dunn's multiple comparison test and considering a significance level of 5% ($p<0.05$).

Results

Animal and soleus muscle weights

This analysis showed a weight decrease in the animals that received nandrolone decanoate with (END: 443 ± 30.94) or without (SND: 485 ± 34.10) physical exercise ($p<0.05$) (Table 1). There was no significant difference in the weight of the soleus muscle in all the experimental groups, regardless of ND use (Table 2).

Laser scanning confocal microscopy

All the groups presented a homogeneous fluorophore response in which acetylcholine receptors were highlighted.

This analysis demonstrated that NMJ postsynaptic region was intact. In the sedentary groups, the NMJs were grouped in small islands. In the exercise group, the acetylcholine receptors had a distribution in continuous branches (Figures 2A, 2C, 2E and 2G).

Morphological and morphometric analysis of NMJs

The identification of NMJs revealed a homogeneous distribution in the middle third of the soleus muscle. Most of NMJs were aligned along the long axis of the muscle fibers. No morphological changes were seen in the groups that were studied (Figure 2 B, D, F and H), and it was statistically demonstrated using the morphometric analyses of NMJs.

Figure 2. I depicts the means of the maximum diameters of NMJs of the different groups. This analysis showed no significant differences in the diameters of NMJs with or without exercise, and regardless of ND use.

Ultrastructural analysis of NMJs

The ultrastructure of NMJ associated with soleus muscles demonstrated myelin presence located in the axon terminals of the

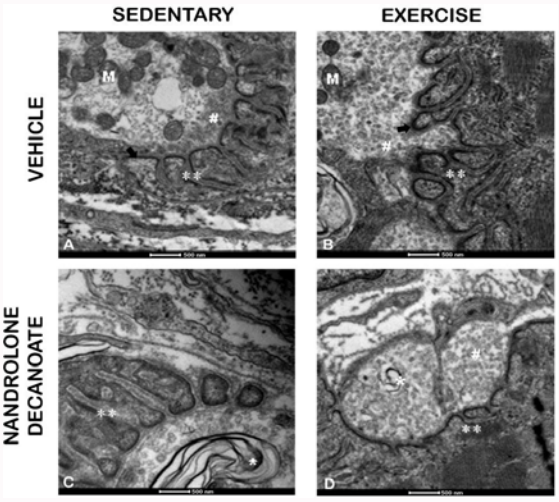

Figure 3: Transmission electron microscopy of NMJs of soleus muscles of all groups. Mitochondria (M), Synaptic vesicles (#), Junctional folds (**), Myelin figures (*), active zone (arrow).

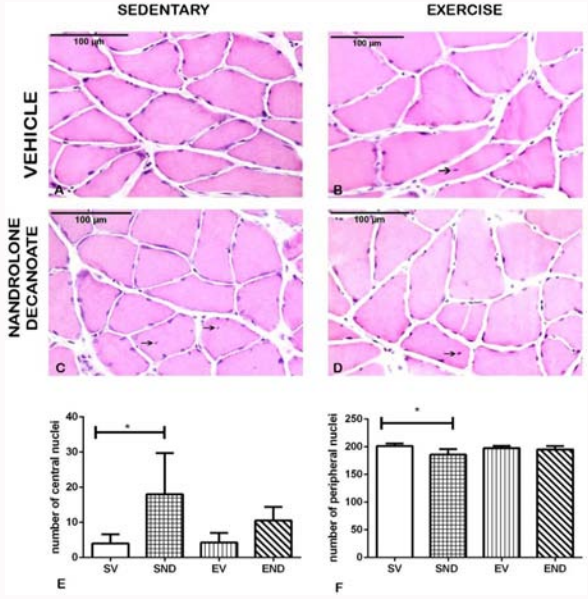

Figure 4: A-D: Photomicrographs of cross-sections of soleus muscle of the experimental groups stained with HE. Arrow: Fibers with central nuclei. E: Means and standard deviation of central nuclei number of soleus muscles. F: Means and standard deviation of peripheral nuclei number of soleus muscles. Statistical results were obtained by a two-factor variance analysis, which was complemented with a Tukey's multiple comparisons test and it was considered a significance level of 5 %. *p<0.05.

groups that received ND (SND and END).

The postsynaptic membrane, which is limited deeply by the synaptic cleft, had a few, ill-defined junctional folds with variable arrangements and dimensions containing no alterations or spaces between them.

The remaining morphology of NMJ showed no differences in the groups. The axon terminals were arranged in synaptic gutters that were sometimes shallow or deep with varied amounts of synaptic vesicles and mitochondria. The presynaptic membrane had electron-dense regions that corresponded to the active zone opposite to the apex of postsynaptic membrane junctional folds (Figure 3).

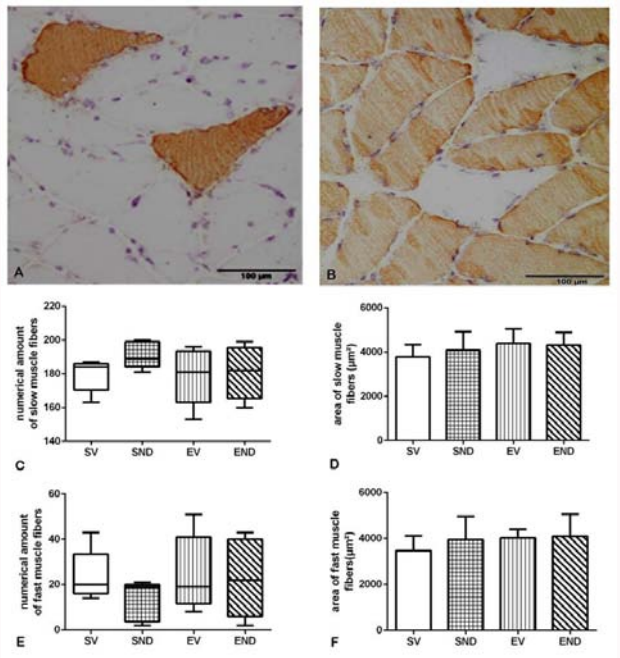

Figure 5: Histological cross-sections of soleus muscle fibers; immunohistochemistry for fast-twitch (A) and slow-twitch (B) fibers. C: Box plot graphic of the slow muscle fiber numerical amount in soleus muscle. D: Means and standard deviation of slow muscle fibers area (µm²). E: Box plot graphic of fast muscle fibers and numerical amount in soleus muscle. F: Means and standard deviation of fast muscle fibers area (µm²). Bars and vertical columns represent averages and standard deviations, respectively. Statistical results obtained by two-factor analysis of non-parametric variance was complemented with Dunn's multiple comparison test for numerical amount of slow and fast fibers and a two-factor variance analysis, which was complemented with a Tukey's multiple comparisons test for the areas of slow and fast fibers, both with a significance level of 5%. *p<0.05.

Morphological and morphometric analysis of muscle fibers

In all the groups, the fibers presented a polygonal shape as well as a preserved endomysium and perimysium (Figure 4A-4D). In the SND and END animals, some fibers presented central nuclei that were confirmed by statistical analysis.

There was an increased number of central nuclei in SND group compared to SV group (SV: 4 ± 2.5 and SND: 18 ± 11.75; p=0.0426) and decreased peripheral nuclei in SND group compared to SV group (SV: 201.3 ± 4.5 and SND: 185.8 ± 9.9; p=0.0261) (Figure 4E, 4F).

Immunohistochemistry analysis (fast and slow twitch fibers)

All groups were stained positively by immunohistochemistry for fast and slow MHC (Figure 5A, 5B).

In the slides where the antibody against slow myosin was used, type I fibers (slow-twitch) were stained, while type II fibers (fast-twitch) did not react to chromogen. In the slides where a fast-myosin antibody was used, type II fibers (fast-twitch) were stained, while type I fibers (slow-twitch) were not. All experimental groups had no changes in the number of slow-twitch or fast-twitch fibers regardless of ND use (Figure 5C, 5D).

The data demonstrated that there was no significant difference both in slow-twitch and fast-twitch fiber areas in all groups regardless of ND use (Figure 5D, 5F).

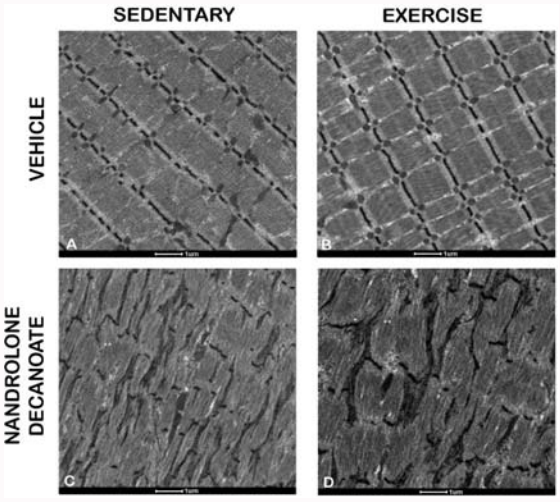

Figure 6: Transmission electron microscopy of soleus muscle fibers. Disorganized sarcomeres (**).

Ultrastructural analysis of muscle fibers

Most of the groups presented a normal sarcomere pattern containing organized myofibrils with preserved organelles by a longitudinal preparation of the muscle fibers. Animals that received ND (SN and END) presented focal areas of injury as well as disorganized sarcomeres and central nuclei (Figure 6).

Discussion

Aging process is associated with progressive loss of muscle mass, strength, and a decline in neurophysiological functions. The relationship between age and NMJ is extremely important in the musculoskeletal impairment that occurs with aging. However, whether alterations in the NMJ precede or follow the decline both in strength and muscle mass still need to be clarified. Many factors, such as mitochondrial dysfunction, oxidative stress, inflammation, changes in muscle fibers innervation, and mechanical properties of motor units, play an important role in NMJ degeneration.

The objective of this study was to investigate whether the association of exercise with ND supraphysiological use during youth prevented neuromuscular age-related alterations. The main findings in ND animals were decreased body weight, focal lesions in myofibrils, disorganized sarcomeres and increased central nuclei at the ultrastructural level as well as altered NMJ junctional folds, myelin figures in axon terminals, and nAChRs organized in islands. In animals that performed exercise, the normal pattern of receptors was maintained in continuous branches. Most of the observed changes were associated with sarcopenia and muscle changes in elderly muscles and NMJ [19].

Muscle mass decrease has been described as an expected pattern for aging animals. It has been connected to sarcopenia [20], and physical activity and metabolic enzymes reduction [21,22].

Binayi et al. [23] who studied Wistar rats, also found weight loss in the groups using ND associated with exercise when they were compared to controls.

The NMJs had similar morphometry in all the studied groups. The alterations of NMJs associated with aging have been demonstrated [24]. Considering that the NMJ morphology varies strikingly among the muscle fibers types according to the kind of fiber they are associated [25], in this study the set of NMJs was analyzed independently of the muscle fibers types. It might be masking these possible alterations.

According to Deschenes et al. [26] along with aging process, NMJs pre-and postsynaptic components remain unchanged, even when NMJ of muscles with different patterns of neuromuscular activity are examined. Therefore, even resistance training does not seem to change any presynaptic component of aged animals NMJ [27].

Pratt et al. [28] stated that physical exercise has a positive effect on the maintenance and regeneration of NMJ, even relative to aging changes. The molecular mechanisms of this process could be leveraged in therapeutic possibilities, which would lead to a decrease in the effects caused by several factors, including aging.

In the ultrastructural analysis of NMJs, myelin figures were present in the axon terminals in groups that received ND. Ozaki et al. [29] studied the changes caused by diabetes associated with aging and also found myelin figures in the fibers. However, the NMJs and capillaries were intact. The myelin figures are entangled membranous structures comprised of phospholipids, which may be replacing groups of dead cells [30].

In all experimental groups, synaptic cleft depth was limited, and the postsynaptic membrane had a few ill-defined junctional folds. Similar results were found by Valdez et al. [31] and Gillon et al. [24] demonstrated that with aging, the postsynaptic folds length decreased in size. Itou et al. [32] found in 30- and 34-month-old mice a nerve terminals area decrease and a loss of synaptic vesicles in addition to junctional folds reduction.

Deschenes noted that with aging [33], NMJs present branches in presynaptic nerve terminals affected the distribution and reception of neurotransmitters in post-synaptic sites. This remodeling has been added to neurophysiological changes. Using confocal microscopy, an island-like pattern was observed in the sedentary animals. Valdez G et al. [31] using α- bungarotoxin, while studying the structural changes of aged mice compared to young animals, also noted that nAChRs of aged rats were often fragmented into small islands. Ferretti et al. [34] when analyzing dystrophic muscles of mdx mice, found the same nAChRs pattern which was associated with utrophin, a protein that acts on the anchoring of nAChR in cytoskeleton.

Li et al. [35] used a time longitudinal analysis to demonstrate that the main structure of NMJ remained stable for many months but it may change suddenly with remodeling/fragmentation of postsynaptic receptors in several aggregates of small nAChRs. That result would explain the NMJs homogeneous morphological and morphometric patterns found in this study using non-specific esterase, although confocal microscopy showed an island- like pattern of the receptors.

In this study, the animals that exercised, with or without ND, presented a pattern of receptors as continuous branches, and normal nAChRs distribution pattern was maintained in adulthood [36,37].

The ultrastructural analysis of the muscle fibers of the SND group confirmed the morphological findings of HE related to the central nuclei. In addition, focal lesions were present as well as signs of discontinuation in the sarcomeres. Ozaki et al. [29] found a similar morphology, describing myofibrillar disorientation, and central nuclei increased number in soleus muscle of aged rats with or without diabetes. It was suggested that the discovered alterations were linked to age-related changes in the fibers.

An important toxic effect of ND was described in rats after

exercises, but such an effect is masked when associated with physical exercise. These effects revealed a safety margin reduction of synaptic transmission in animals not submitted to exercise [38].

In this study the animals that performed exercises had a pattern of nAChRs in continuous branches, and in all the other animals the "island" pattern was present. The morphometric and quantitative pattern of slow- and fast-twitch fibers remained stable in all groups. Central nuclei and focal areas of injury as well as myofibrillar disorganization were observed in the animals that got ND.

Conclusion

It can be concluded that the changes observed in this study were related to aging process and that physical exercises performed during youth maintained the structural pattern of nAChRs. On the other hand, ND did not prevent age- related morphological changes in the neuromuscular system. Physical exercises are and will always be beneficial to the neuromuscular system.

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