Supplementation with fish oil reduces morphological aspects of muscle damage induced by intense exercise in rats

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ABSTRACT
AIMS: To investigate the effects of resistance exercise and fish oil intake on muscle morphology in Wistar rats.
METHODS: Forty-eight animals that performed resistance exercise were initially divided into two groups. One group did not take fish oil and the other group took fish oil. The animals of the second group underwent training and took fish oil for eight weeks. At the end of the last resistance exercise session, the 48 rats were organized into six subgroups of eight each, according to the time gap (12, 24 or 48 hours) elapsed until the gastrocnemius muscle withdrawal procedure. At each established time after the last resistance exercise session, the gastrocnemius muscle was removed for morphological analysis.
RESULTS: Skeletal muscle cells of the animals that did not receive fish oil presented higher scores of edema, especially those from the groups that had their muscles withdrawn at 24 and 48 hours of time gap. As for the group that took fish oil, we observed a smaller amount of inflammatory infiltrate and reduced areas of necrosis compared to animals that exercised without the use of fish oil, at all post-exercise time gaps.
CONCLUSIONS: Fish oil intake attenuated morphological changes in muscle tissue after high-intensity exercises.

KEYWORDS: inflammation; exercise; muscle tissue; polyunsaturated fatty acids; nutrition.

RESUMO
OBJETIVOS: Investigar os efeitos do exercício resistido e da ingestão de óleo de peixe na morfologia da fibra muscular em ratos Wistar.
MÉTODOS: Quarenta e oito animais que realizaram exercício resistido foram divididos inicialmente em dois grupos. Um dos grupos não recebeu óleo de peixe e o outro grupo ingeriu óleo de peixe. Os animais do segundo grupo realizaram o treinamento e ingeriram óleo de peixe por um período de oito semanas. Ao final da última sessão de exercício resistido os animais foram organizados em seis subgrupos de oito cada, segundo o intervalo de tempo (12, 24 e 48 horas) transcorrido até o procedimento de retirada do músculo gastrocnêmio. Em cada tempo determinado após a última sessão de exercício resistido, o músculo gastrocnêmio foi retirado para análise morfológica.
RESULTADOS: As células do músculo esquelético dos animais que não receberam óleo de peixe apresentaram escores maiores de edema, especialmente as dos grupos que tiveram os músculos retirados em 24 e 48 horas. No grupo que ingeriu o óleo de peixe observou-se menor quantidade de infiltrado inflamatório e áreas de necrose reduzidas em comparação com os animais que se exercitavam sem o uso de óleo de peixe, em todos os intervalos de tempo pós-exercício.
CONCLUSÕES: A ingestão de óleo de peixe atenuou as alterações morfológicas no tecido muscular após exercícios de alta intensidade.

DESCRITORES: inflamação; exercício; tecido muscular; ácidos graxos poli-insaturados; nutrição.
INTRODUCTION

Nutritional supplements for reaching recommended daily needs have been widely used in different sports modalities. Natural components of numerous foods have physiological effects and some of them are considered useful to improve sports performance or to prevent injury [1, 2].

Some studies suggest that the use of creatine may reduce muscle damage or improve recovery after intense exercise. Plasma levels of the enzymes creatine kinase and lactate dehydrogenase, and inflammatory indicators such as prostaglandins, tumor necrosis factor alpha (TNF-α) and C-reactive protein were decreased with the use of this supplement [3-6]. Branched chain amino acid supplementation was considered a potential nutritional strategy to avoid or at least alleviate exercise-induced muscle damage or its consequences [7, 8].

Among other food components that have physiological action and are considered a functional substance, the compounds rich in polyunsaturated fatty acids (PUFA) of type omega-3 have aroused the researchers’ interest and also caught considerable attention concerning nutritional support to maintain muscle fiber integrity and damage inhibition after exercise [2, 9-11]. This attention is related to the anti-inflammatory and immunomodulatory properties conferred to these fatty acids [12, 13].

Omega-3 PUFA have to be obtained in adequate quantities for human nutrition, since mammals do not synthesize this type of fat. They are found in high concentrations, in the form of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), in several foods, particularly in some cold and deep water fish, such as mackerel, salmon, and tuna [2, 9, 14-16]. Among the beneficial effects of fish oil, its action on decreasing the synthesis of potent chemical mediators of inflammation derived from arachidonic acid, such as prostaglandin, thromboxane, prostacycline and leukotriene, ensuring anti-inflammatory effect, is highlighted [12, 13, 17].

The consumption of compounds rich in omega-3 PUFA may be associated with decrease of muscle damage indirect markers [9-11, 18-20]. Knowledge about these benefits is important for athletes, who are constantly subjected to muscular damage, especially when they perform high-intensity eccentric exercises. For Haidamus [21], the use of foods rich in omega-3 PUFA, EPA and DHA by athletes may minimize the effects of the inflammatory process on the injured muscle by decreasing the synthesis of potent chemical mediators of inflammation and, therefore, reducing their recovery time and improving their responses to high-intensity exercises [22]. However, data are still conflicting as to whether omega-3 PUFA suppress the inflammatory response after high-intensity exercise. Thus, this study aimed to test the hypothesis of possible anti-inflammatory effect and contribution to muscle damage prevention of omega-3 PUFA (EPA and DHA) derived from fish oil after high-intensity exercises. This article also aims to investigate changes in the skeletal muscle in rats submitted to exercise with weights and fish oil intake.

METHODS

Experimental animals

A total of 48 male Wistar rats (Rattus norvegicus) with a mean age of 120 days, weighing between 220g and 270g, provided by the Animal Model Experimentation Center (CEMA) at the University of Marilia/UNIMAR were used. The animals were transferred to the experimental area and kept in climatized environment with temperature control (between 22°C to 24°C), and light and dark cycle of 12/12 hours. The animals were kept in polyethylene cages (four animals per cage) for eight days and received NuviLab® commercial pelleted food (by Nuvital) and water ad libitum.

This study was approved by the Research Ethics Committee of the Federal University of São Paulo (UNIFESP), with protocol number 0354/12. During the experiment, the animals were treated according to the UNIFESP document “Guide for the Care and Use of Experimental Animals”. The study complied with the Brazilian guidelines for the care and use of animals for scientific and didactic purposes.

Experimental groups

All the animals were kept in collective cages and performed resistance exercise three times a week for eight weeks. They were initially divided into two groups of 24 animals each: the resistance exercise group (EX) received no fish oil; and the resistance exercise and fish oil group (EXFO) received fish oil after each resistance exercise session. At the end of the last resistance exercise session, the animals of the two groups were divided into subgroups with eight
animals each, according to time (12, 24 and 48 hours) for the gastrocnemius muscle withdrawal procedure. At the established time after the last resistance exercise session, the gastrocnemius muscle was removed for morphological analysis.

**Resistance exercise protocol**

Exercise consisted of vertical jumps to the surface in a pool 50 cm height by 25 cm diameter, filled with 35 cm of heated water (30°C). To perform the exercises, an additional load was attached to the animal thorax [23]. The exercise protocol consisted of 10 repetitions for four sets with a 60 s rest among sets, three times a week for eight weeks.

The load adjustments were made weekly, considering the body weight, which was controlled at the same frequency. To reduce stress, the animals were adapted to the aquatic environment for one week before the beginning of the protocol. This adaptation consisted of resistance exercise sessions with load (30% of body weight) attached to the thorax, for five consecutive days. After the adaptation week, the experimental resistance training protocol was started, as described by Renno et al. [24] and Secchi et al. [25]:

- First and second weeks: 4 series of 10 jumps, with overload of 50% of the body weight;
- Third and fourth weeks: 4 series of 10 jumps, with overload of 60% of the body weight;
- Fifth and sixth weeks: 4 series of 10 jumps, with overload of 70% of the body weight;
- Seventh and eighth week: 4 series of 10 jumps, with 80% overload of the body weight;

This protocol was chosen based on the similarity of number of sets, repetitions, rest period and strength training frequency with resistance training protocol applied in humans.

**Fish oil administration**

Animals from the EXFO group received supplementation of 2 g/kg/day of fish oil as recommended by Simopoulos [26], containing omega-3 PUFA, 50 mg EPA and 50 mg DHA (Ativus Farmacêutica Ltda, Valinhos, São Paulo Brazil). Administration was performed after eight-week resistance exercise sessions using a stomach tube adapted to a five miligram syringe (gavage method).

**Muscle collection and histological analysis**

After 12, 24 and 48 hours from the last resistance exercise session, the animals were anesthetized with Hypnol® (pentobarbital sodium). The entire gastrocnemius muscle was removed with right lower limb posterior region incision and weighed. The gastrocnemius muscle was chosen due to a greater proportion of type II muscle fibers [27], recruited in activities with a high speed of contraction and great strength per motor unit. The samples were put on microscope slides and stained with hematoxylin and eosin for histological analysis under light microscopy.

**Microscopic analysis and photomicrography**

Histological sections were evaluated by an experienced professional, without prior knowledge of the experiment, using a Nikon® Eclipse E-200 microscope. In order to evaluate the degree of damage process, one slide per animal was analyzed. For qualitative analysis, each slide was analyzed in 20 fields with magnification from 40 to 100 times in different foci in areas identified as “hot spots”. For quantitative analysis, the intensity of the neutrophilic infiltrate (inflammatory response) edema (fiber dissociation) and necrosis (sarcolemma destruction) were evaluated in the histological sections, and a system of parameters (scores) was established by Brasileiro et al. [28] and is described in **Chart 1**. Arithmetic means of values found in the 20 fields counted in each slide, corresponding to one animal in each group, were calculated for each parameter.

**Chart 1. Microscopy parameters of muscle damage and damage intensity degree.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Grade 0 (absent)</td>
</tr>
<tr>
<td>Edema</td>
<td>Absent</td>
</tr>
<tr>
<td>Neutrophil infiltrate</td>
<td>Absent</td>
</tr>
<tr>
<td>Necrosis</td>
<td>Absent</td>
</tr>
</tbody>
</table>

From Brasileiro et al. [28].
Statistical analysis

Data were expressed as mean and standard deviation. The analysis was initially performed by the Kolmogorov-Smirnov normality test and after normality was checked, Variance Analysis (ANOVA) was performed, complemented by Tukey’s multiple comparisons test. The null hypothesis significance level for rejection was 0.05. For all tests, the program Graphpad Prism 5.0 (San Diego, California, USA) was used.

RESULTS

As for semi-quantitative analysis, we observed that the distance of muscle fibers presented higher scores in the groups corresponding to 24 and 48 hours post-resistance exercise. The animals that took fish oil had statistically more favorable dissociation scores of muscle fibers in comparison with the ones that were submitted to exercise and did not take fish oil (Figure 1A).

As for inflammatory infiltrate, the animals that received fish oil presented more representative scores for mild to moderate degree, while the animals that did not receive fish oil presented more scores that were moderate to intense, featuring numerous cells per field (Figure 1B).

The animals belonging to the groups that took fish oil presented at most one fiber with necrosis, while the animals that exercised and did not take fish oil had at least two fibers with necrosis per field (Figure 1C).

Qualitative analysis of muscle fibers demonstrated that the skeletal muscle cells of the group that did not take fish oil presented intense edema among the fibers and small neutrophil infiltration (Figure 2A). Necrosis, neutrophilic infiltration and edema were manifested by the gap of muscle cells due to interposition of interstitial fluid (Figure 2B). A large focus of necrosis characterized by muscle cells of hyaline staining without nucleus in their usual topography was identified (Figure 2C). In sections in which the exercised animals took omega-3 PUFA, moderate degree of intercellular edema and the focal and sparse presence of acute inflammation (neutrophils) were identified (Figure 2D). A moderate edema was observed among the fibers and moderate neutrophil infiltration (Figure 2E). Despite the area of necrosis characterized by hyalinized muscle tissue and mild edema, fibers with normal contours were observed (Figure 2F).

Figure 1. Mean and standard deviation of muscle fiber dissociation (A), muscle fiber neutrophilic infiltrate (B) and muscle fiber necrosis (C) analysis scores for subgroups of Wistar rats with or without fish oil intake, all of which were subjected to resistance exercise.

EX12: Group that did not take fish oil; muscle withdrawal procedure 12 hours after the last exercise session.
EXFO 12: Group that ingested fish oil; muscle withdrawal procedure 12 hours after the last exercise session.
EX24: Group that did not take fish oil; muscle withdrawal procedure 24 hours after the last exercise session.
EXFO 24: Group that ingested fish oil; muscle withdrawal procedure 24 hours after the last exercise session.
EX48: Group that did not take fish oil; muscle withdrawal procedure 48 hours after the last exercise session.
EXFO 48: Group that ingested fish oil; muscle withdrawal procedure 48 hours after the last exercise session.
Figure 2. Photomicrographs of the gastrocnemius muscle histological sections of Wistar rats with or without fish oil intake, all of which were subjected to resistance exercise.
DISCUSSION

This study found fish oil effects on morphological aspects of muscle fibers after high-intensity exercise. The mechanisms of injury are still studied and are controversial. It has been commonly assumed that mechanical stress causes several cellular changes such as increase of the cytoplasmic calcium concentration, which causes proteolysis and lipolysis in the muscle cells’ membrane, since it is able to activate specific enzymes sensitive to its high concentration. Furthermore, mechanical stress may affect the excitation-contraction process [29]. Intracellular accumulation of calcium favors phospholipase A2 activation and induces production of prostaglandins, leukotrienes and free radicals (powerful chemical mediators of inflammation), mainly from arachidonic acid in the membrane structure [30].

Different from our experimental exercise model, Garcia et al. [31] submitted rats for 28 days to continuous aerobic training with an overload of 5% of body weight and supplemented with fish oil. Histological analyzes of the soleus muscle of the animals supplemented with fish oil showed that the fibers had no increase in endomysial tissue, peripheral nuclei with reduced phagocytosis, and less pronounced polymorphism. Histological patterns of muscle damage were observed in the specimens from animals that trained without fish oil supplementation. In that study, the authors pointed out that the use of fish oil had a protective effect against muscular injuries [31]. In the same way, despite the use of different techniques, our study observed intense edema, neutrophilic inflammation and foci of necrosis in groups that performed resistance exercise and did not take fish oil. According to the classification of damage established for our study, the frequency of animals that presented moderate and intense edema was higher in groups that exercised without the supplementation with fish oil. Most of the animals belonging to the groups that received fish oil had no neutrophilic infiltrate. Some degree of necrosis was observed more frequently in animals that exercised without the use of fish oil.

It is important to notice that higher systemic omega-3 PUFA levels correlate with its increased concentration in the muscle cell membrane, increasing elasticity, flexibility and reduced risk of damage after intense exercise [32, 33]. Possibly, the strategy of fish oil intake used in our experimental model contributed to the lower amplification of damages.

According to Tsuchiya et al. [34], omega-3 PUFA incorporation into the muscle cells membrane relieves tissue inflammation, thus suppressing the rupture of neuromuscular junction and the post-synaptic electrical transition. After exercise-induced muscle damage, a local inflammatory response is triggered, and accompanied by some edema. Neutrophils migrate to the damage site and the fight against the damaged tissue begins. Between 6 and 12 hours, monocytes accumulate in the site and a peak concentration occurs in about 48 hours after exercise. Monocytes are converted into macrophages, which synthesize large amounts of prostaglandin E2 [35].

Inflammation is a physiological response to tissue damage. It is primarily characterized by an increase in the cytokine expression, such as TNF-α, interleukin (IL)-1β and IL-6 [36]. Cytokines can trigger release of acute phase proteins and inflammatory mediators, such as leukotriene and prostaglandin. In a recent study with male subjects, Jakeman et al. [37] have shown that acute ingestion of fish oil may inhibit elevation of serum cytokines and markers of muscle damage after jumping exercises. Muscle recovery after exercise influences training adaptations. Relatively recent studies corroborate our findings, demonstrating the considerable effect of omega-3 PUFA on muscle recovery and repair on muscle fiber damage [34, 38].

Mickleborough et al. [39] reported attenuation of muscle injury blood markers (troponin I, myoglobin, creatine kinase) and inflammation (TNF-α) associated with the use of omega-3 PUFA-rich supplement after muscle injury induced by intense exercise. A study developed with wheelchair basketball athletes concluded that the use of fish oil supplementation rich in DHA prevented muscle injury, as well as changes in the profile of inflammatory mediators, neutrophil function and necrosis. Unlike this study, the subjects were supplemented daily for 30 days with 3 g of fish oil [40].

Brouard and Pascaud [41] studied inflammatory mediators in rats with diet composed of fish oil (rich in EPA and DHA), and reported a statistically significant decrease in inflammatory mediators. This can be explained because the anti-inflammatory effects of omega-3 PUFA are due to the decrease in the arachidonic acid content of the membranes resulting in the synthesis of eicosanoids derived from the diminished omega-6 PUFA [42-46].

Russ et al. [47] investigated the effect of eight weeks of fish oil supplementation on attenuation of contusion injuries in rats. The contusion model used by the authors produced significant muscle impairment, and the study showed that fish oil intake did not improve nor worsen the lesion. However, the authors observed an increase in myogenic factors and
a decrease in mitofagia factors that may suggest fish oil as a potential strategy in post-injury recovery [47].

The slightest changes observed in the muscle fibers of fish-eating animals may be related to the fish oil anti-inflammatory properties, by reducing pro-inflammatory mediator synthesis, and preventing inflammatory response exacerbation to muscle damage process induced by physical stress due to resistance exercise. These findings can be explained by the fact that omega-3 PUFA rich compounds such as fish oil compete with arachidonic acid, reducing production of pro-inflammatory compounds (prostaglandin E2, thromboxane A2, prostaglandin I2, leukotriene 4) resulting from this acid, and favoring less active compound production (leukotriene 5, prostaglandin E3, thromboxane A3, prostaglandin I3) with anti-inflammatory potential derived from omega-3 PUFA [48, 49].

The production of leukotriene 5 was demonstrated in macrophages of rats fed with fish oil [50] and human neutrophils supplemented with fish oil for several weeks [51-53]. Eicosanoids derived from omega-3 PUFA are much less biologically active than those produced from arachidonic acid are. Omega-3 derived leukotriene B5 have 10 to 100 times less neutrophil chemoattractant potential compared to leukotriene B4 [54, 55].

We lost four experimental animals belonging to the group that took the fish oil. As a limitation of the study we did not investigate animal deaths, but we can not relate them to any adverse effects of fish oil. Studies report few adverse effects with the use of omega-3 PUFA-rich supplements. Doses greater than 3 g/day in humans have been related to higher risks of hemorrhagic events due to a longer bleeding time [11, 56, 57]. Simopoulos [26] recommends that most athletes, especially at the leisure level, should include in their diet about 1-2 g/day of omega-3 PUFA.

We concluded that the physical exercise model applied in this study produced morphological changes in muscle cells and fish oil intake seems to be a useful resource for the muscle protection or recovery, since it attenuated the changes, demonstrated by smaller scores of edema, neutrophilic infiltrate and necrosis in muscle tissue.

NOTES

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Conflicts of interest disclosure

The authors declare no competing interests relevant to the content of this study.

Authors’ contributions

All the authors declare to have made substantial contributions to the conception, or design, or acquisition, or analysis, or interpretation of data; and drafting the work or revising it critically for important intellectual content; and to approve the version to be published.

Availability of data and responsibility for the results

All the authors declare to have had full access to the available data and they assume full responsibility for the integrity of these results.

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