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muscle wasting in a collagen-induced arthritis model**

Adriano Rostirolla Linhares

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Orientador: Prof. Dr. Ricardo Machado
Xavier

Co-orientador(a): Dra. Lidiane Isabel
Filippin

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MODERATE AEROBIC EXERCISE DOES NOT PROTECT FEMALE MICE FROM MUSCLE WASTING IN A COLLAGEN-INDUCED ARTHRITIS MODEL

Adriano Rostirolla Linhares^{1,2}, Jordana Miranda de Souza Silva^{1,2}, Paula Ramos Viacava², Paulo Vinicius Gil Alabarse², Vivian de Oliveira Nunes Teixeira², Ricardo Machado Xavier², Lidiane Isabel Filippin²

¹Universidade Federal do Rio Grande do Sul

²Laboratório de Doenças Autoimunes e Infecciosas – Hospital de Clínicas de Porto Alegre

Avenida Ramiro Barcelos, 2350, Bairro Rio Branco

CEP 90035-903 Porto Alegre, RS, Brazil

email: adrianorostirolla@gmail.com

phone number: (51) 3359 8837

Abstract

Background

Many rheumatoid arthritis (RA) patients undergo loss of muscle mass, which is a feature that leads to increased morbidity and mortality. Although the causative mechanisms of muscle wasting in RA are not fully understood, it has been suggested that physical exercise may be useful in reverting this condition. In this study, we aimed to evaluate the effect of moderate aerobic exercise upon skeletal muscle of mice subjected to collagen-induced arthritis (CIA).

Methods

CIA mice were subjected to aerobic exercise in a treadmill 5 days per week over 4 weeks. Sedentary CIA mice were used as control. Clinical severity of the joints and paw oedema were assessed daily; spontaneous exploratory locomotion and body weight were evaluated weekly. Tibialis anterior muscles were analyzed for weight and myofibers cross-sectional areas; gastrocnemius were used for total proteins measurement and western blot analysis of myogenin and Pax-7 expressions.

Results

Arthritis score ($p = 0,08$), paw oedema ($p = 0,06$), tibialis weight ($p = 0,66$), gastrocnemius weight ($p = 0,20$) and tibialis cross-sectional areas ($p = 0,71$) were not significantly different between groups, suggesting that exercise was not able to decrease muscle wasting. Distances coursed in spontaneous locomotion was not different between groups ($p=0,36$), most likely because there was not muscle mass improvements. Quantifications of myogenic transcription factors myogenin ($p = 0,71$) and Pax-7 ($p = 0,27$) were also of no statistical difference between groups.

Conclusions

In this model of CIA, moderate aerobic exercise did not cause increase damage to joints, but was not able to protect female mice from muscle wasting. Further studies are needed to evaluate influences of androgens upon muscle growth in CIA mice.

Keywords: collagen-induced arthritis – muscle wasting – exercise – myogenic regulatory factors

1 Introduction

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease of unknown etiology (1). Progression of the disease usually leads to joint pain and reduced muscle strength and endurance (2), which combined with ensuing joint destruction may cause impaired physical function (3). Most RA patients undergo body composition changes known as rheumatoid cachexia (1). This is a systemic manifestation characterized by a loss of body cell mass mainly on skeletal muscle whereas fat mass remains constant or is increased; these changes in body composition lead to weakness as well as increased risk of cardiovascular disease (4) and metabolic alterations (5), thereby driving most RA subjects to a sedentary lifestyle (3) and resulting in reduced life expectancy (1).

It has long been suggested that proinflammatory cytokines such as tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) have a causative role in muscle wasting (1,6) by means of nuclear factor-kappa B (NF- κ B) pathway activation in myocytes (7). NF- κ B probably induces loss of muscle by activating the ubiquitin-proteasome system and reducing levels of MyoD protein, a transcription factor involved in myogenesis (7). Moreover, myogenin is a transcriptional factor that was shown to be decreased in cancer and experimental cirrhosis (8,9) and in muscles of cancer cachectic patients (10).

So far, there is no standard therapeutic approach for muscle wasting in RA (4,11). In the last years, the positive role of exercise on muscle wasting in RA has been a matter of debate. Collagen-induced arthritis model (CIA) in mice has been extensively used as a RA model (12), demonstrating as a useful tool for studying joint pathological features. By contrast, the effects of physical exercise upon muscles of animals subjected to CIA had not been put in practice until this time, according to our knowledge.

In the present study, we evaluated muscle mass, myofibers cross-sectional areas, spontaneous locomotion and expression profiles of some major myogenic regulatory factors (Pax-7 and myogenin) in muscles of collagen-induced arthritis (CIA) mice subjected to moderate aerobic exercise. Therefore, we aimed to analyze the effects of aerobic exercise in CIA mice upon muscle properties and levels of two proteins linked to regulation of myogenesis.

2 Materials and methods

2.1 Mice and experimental groups

Sixteen female DBA/1J mice at 8 to 12 weeks of age were used. They were kept under 12-h light/dark cycle, at 20 °C with controlled humidity, and had free access to standard pellet diet and water. The animals were randomly divided into two experimental groups: collagen-induced arthritis sedentary (CIA) (n = 8) and collagen-induced arthritis subjected to physical exercise (EXE) (n = 8). All experiments were performed according to the Guiding Principles for Research Involving Animals (NAS) and to the Ethics Committee of Research and Postgraduate Group of the Hospital de Clínicas de Porto Alegre (HCPA).

2.2 Experimental procedures

Arthritis was induced, on day 0, by intradermal injection in the base of the tail of 50 µL of an emulsion comprised of bovine type II collagen (CII, Chondrex, Inc.; 2 mg/ml) dissolved in 0.1 M acetic acid at 4 °C for 12 h and complete Freund's adjuvant (Sigma, St Louis, MO, USA; 4 mg/ml) containing inactivated *Mycobacterium tuberculosis* (strain H37RA, Difco, Detroit, MI, USA). After 18 days, animals received a secondary immunization (booster) with the same concentration of CII emulsified with incomplete Freund's adjuvant (IFA - without *M. tuberculosis*) in another site of the tail, and were sacrificed at 4 weeks after induction of arthritis (adapted from (13)). During the procedures, the animals were anesthetized with inhalation isoflurane (1 ml/ml, Abbott). Tibio tarsal joints were dissected to confirm development of arthritis through histological analysis. Tibialis anterior and gastrocnemius muscles were both dissected for weight and myofibers cross-sectional area assessment, and muscle protein measurements, respectively; muscle weights were normalized to body weights. Body weights were determined weekly starting from booster, and the values were subtracted from the body weight of healthy animals assessed at the time of induction.

2.3 Clinical severity and measurement of edema

Arthritis severity was clinically determined for each paw, daily, according to the following scale of 0 to 4: 0 - no evidence of erythema and swelling; 1 - erythema and mild swelling confined to the tarsals or metatarsals; 2, erythema and moderate swelling of tarsal and metatarsal or tarsal and ankle joints; 3 - erythema and severe swelling extending from the ankle to metatarsal joints; and 4 -

erythema and severe swelling encompassing the ankle, foot and digits, or ankylosis of the limb (13). The most clinical score that a mouse could have reached was 16. The day of arthritis incidence was considered on the first signs of arthritis (when a grade of 1 or more could be visible for the first time in at least one paw). Paw edema was daily assessed by measuring size of latero-lateral height extent of the metatarsus hind paws with a digital caliper.

2.4 Assessment of locomotion

Mice spontaneous exploratory locomotion was evaluated to seek whether this parameter is correlated to muscle mass and arthritis severity. Once-weekly, starting the day of the booster, mice were placed individually inside an acrylic box (Monitor de Atividade IR) of 60 cm X 40 cm. Spontaneous exploratory locomotion of the animals was detected by sensor bars located in the sides of the box during 5 minutes, after 30 seconds of adaptation time (adapted from (12)). Movements were evaluated by a computer software (Insight Equipamentos Ltda®) considering the following parameters: route design, walked distance, mean velocity, resting time, and number of times standing.

2.5 Exercise training

After the onset of arthritis, mice were subjected to moderate physical exercise in a motorized rodent treadmill (developed by engineering staff of HCPA) for 30 min a day, 5 days per week over 4 weeks. The exercise program consisted of five sequentially steps of locomotion performed at different velocities and time durations, as follows: a 5 minutes locomotion at a speed of 9 m/min, 5 minutes at 12 m/min, 10 minutes at 15 m/min, 5 minutes at 12 m/min, and 5 minutes at 9 m/min (adapted from (14)).

2.6 Histological scoring

An histological scoring system (15) was used to evaluate individual joints and measure arthritis severity. Briefly, to assess synovial inflammation, five high-power magnification fields (HMF) were scored for the *percentage of infiltrating mononuclear cells* as follows: 0 – absent, 1 - mild (1–10 %), 2 - moderate (11–50 %), 3 - severe (51–100 %); *synovial hyperplasia* (described by (16)): 0 – absent, 1 - mild (5–10 layers), 2 - moderate (11–20 layers), 3 - severe (>20 layers); *extension of pannus formation based on the reader's impression*: 0 – absent, 1 - mild, 2 - moderate, 3 – severe;

cartilage erosion - percentage of the cartilage surface that was eroded (described by (16)): 0 – absent, 1 – mild (1–10 %), 2 - moderate (11– 50 %), 3 - severe (51–100 %); *bone erosion*: 0 – none, 1 - minor erosion(s) observed only at HMF, 2 – moderate erosion(s) observed at low magnification, 3 – severe transcortical erosion(s).

2.7 Myofibers cross-sectional areas

Tibialis cross-sectional areas were used as a quantitative parameter of muscle atrophy. To estimate transverse section areas of myofibers, we used the software Image-Pro Express (version 5.1.0.12, Media Cybernetics). Each cross-sectioned anterior tibialis muscle was visualized in a microscope (400X), and ten fields were photographed. Then, twenty myofibers from each picture were measured by drawing two perpendicular straight lines crossing the fiber on computer. The mean of these diameters (in micrometer) was used to calculate the transverse section area, and doing so we had an estimation of the myofibers cross-sectional areas.

2.8 Western blot analysis

Western blot analysis of myogenin and Pax-7 were carried out to evaluate differences in muscle regeneration between groups. Gastrocnemius muscle homogenates were prepared and total protein concentration was measured using Bradford assay (17). Blots were quantified using ImageJ 1.46 software, and all quantifications were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression of each animal.

2.9 Statistical analysis

Statistical analysis was carried out using Graph Pad Prism 5 statistical software, considering a level of 5% probability as significant. Differences between groups were analyzed by Student's t test, and two-way ANOVA tests were used to assess differences and interactions between groups. All data are expressed as means \pm standard error of the mean (SEM).

3 Results

3.1 Arthritis severity and body weight

All animals used in the study developed clinically detectable arthritis. Clinical severity of arthritis (figure 1) and paw oedema (figure 2) were not significantly different between groups ($p = 0,08$ and $p = 0,06$ respectively), and likewise histological scoring (table 1). Notwithstanding that, it could be observed a trend for slower disease progression in EXE group, over the first nine days after the onset of disease (figure 1). There was no significant difference between body weight variations between groups ($p = 0,43$) as shown in figure 3, although there seems to be some trend towards larger weights in EXE group from third week on.

3.2 Muscle mass, total proteins and myofibers cross-sectional areas

There were no significant differences in the weight variables of tibialis ($p = 0,66$) and gastrocnemius ($p = 0,20$), and in gastrocnemius total proteins ($p = 0,51$) between groups (figures 4, 5 and 6 respectively). Tibialis cross-sectional areas were larger in EXE group, but of no significant difference between groups ($p = 0,71$), as shown in figure 7.

3.3 Assessment of locomotion

Locomotion averages did not statistically differ between groups ($p = 0,36$) (figure 8). EXE group coursed larger distances than CIA group at the booster and at weeks 1 and 3, whereas CIA group coursed larger distances at weeks 2 and 4.

3.4 Western blot

There were no significant differences in quantification of myogenin ($p = 0,71$) and Pax-7 ($p = 0,27$) between groups, as shown in figures 9 and 10 respectively. Both proteins were detected in lesser quantities in EXE group in comparison to CIA group.

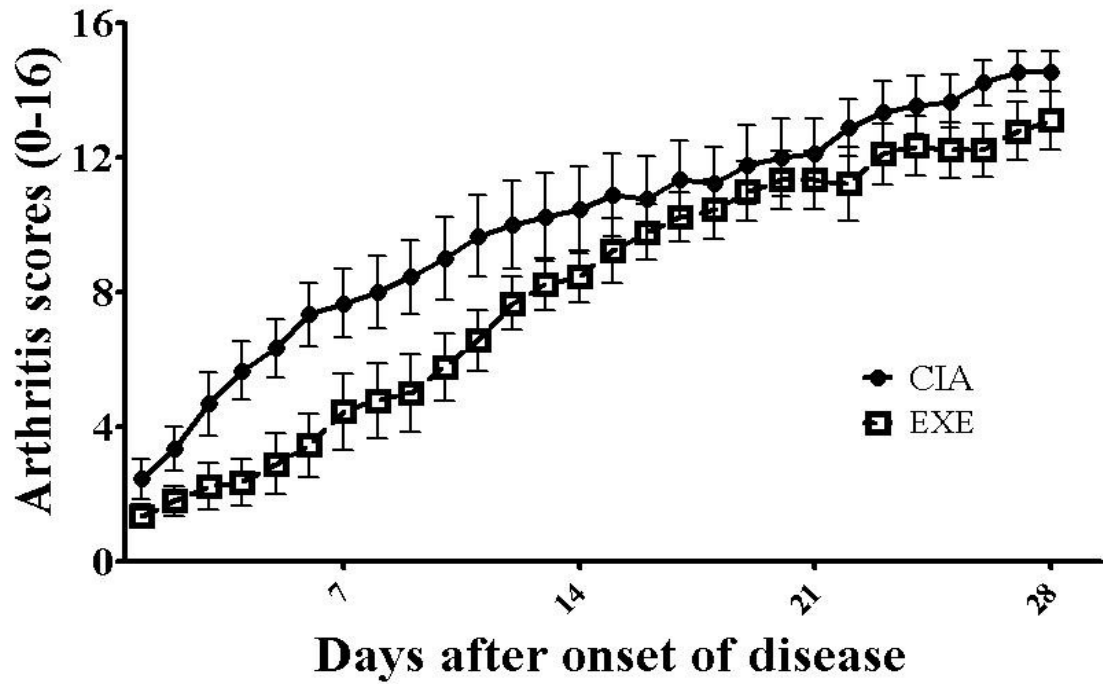


Fig1. Clinical severity of arthritis by clinical score of each paw. CIA = sedentary group; EXE = exercise group. Each value represents the mean of arthritis scores \pm SEM; data were analyzed by two-way ANOVA; $p < 0,05$.

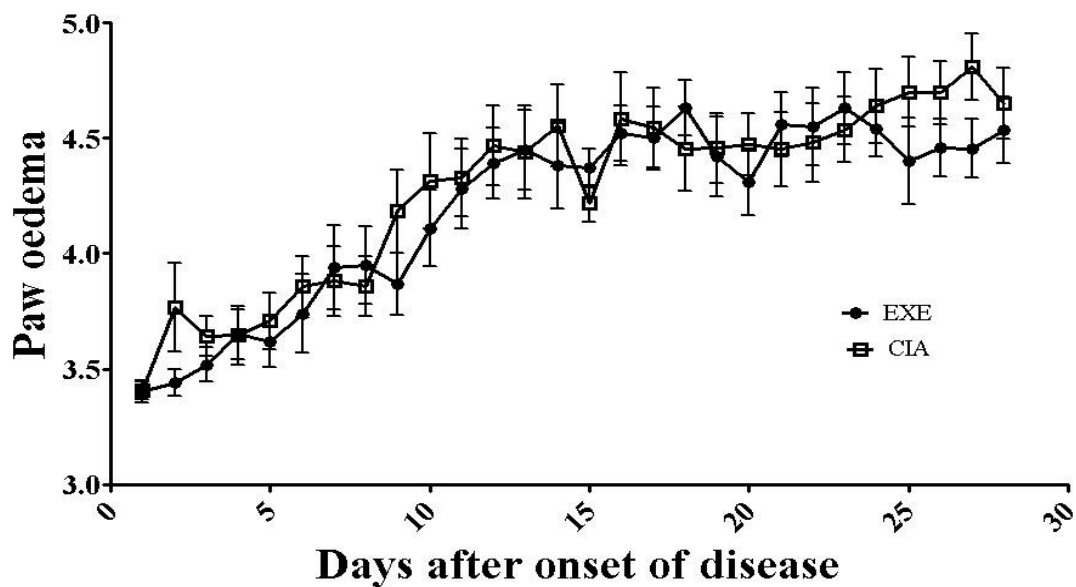


Fig2. Paw oedema by measuring size of latero-lateral height extent of the metatarsus hind paws with a digital caliper. CIA = sedentary group; EXE = exercise group. Each value represents the mean of arthritis scores \pm SEM; data were analyzed by two-way ANOVA; $p < 0,05$.

Table 1. Histological parameters of the joints. Results are expressed as means \pm SEM; data were analyzed by Student's t test; $p < 0,05$.

	CIA	EXE
Inflammation	1,50 \pm 0,60	1,22 \pm 0,80
Hyperplasia	0,07 \pm 0,18	0,28 \pm 0,51
Pannus formation	1,00 \pm 0,38	0,83 \pm 0,79
Cartilage erosion	0,50 \pm 0,38	0,56 \pm 0,58
Bone erosion	0,71 \pm 0,45	0,72 \pm 0,76

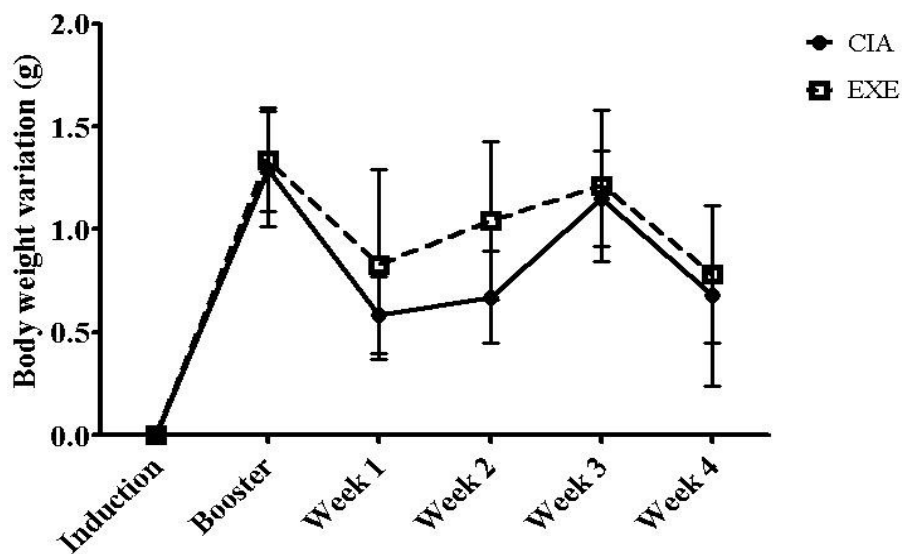


Fig3. Mice body variations (= difference between arthritic and healthy mice). Symbols as in fig. 1. Each value represents the mean of body variations \pm SEM; data were analyzed by two-way ANOVA; $p < 0,05$.

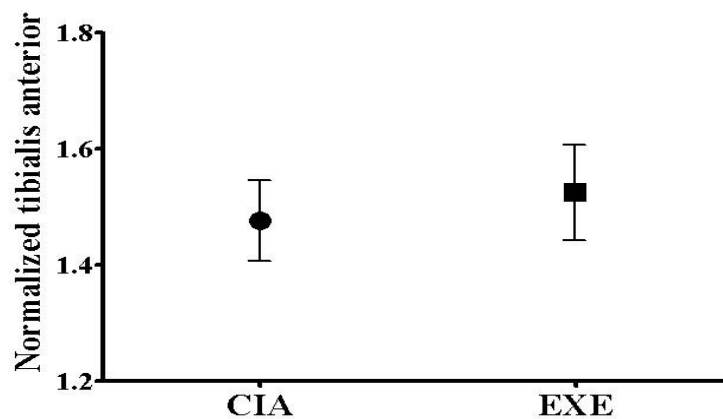


Fig4. Tibialis anterior weights normalized to body weights. Symbols as in fig. 1. Each value represents the mean of normalizations \pm SEM; data were analyzed by Student's t test; $p < 0,05$.

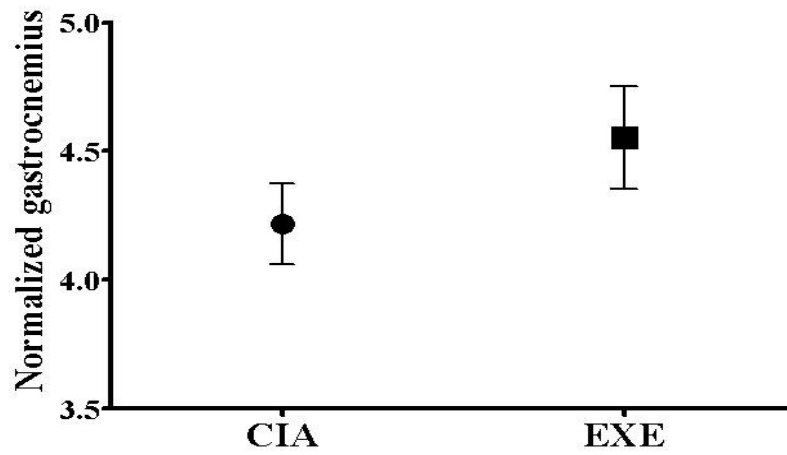


Fig5. Gastrocnemius weights normalized to body weights. Symbols as in fig. 1. Each value represents the mean of normalizations \pm SEM; data were analyzed by Student's t test; $p < 0,05$.

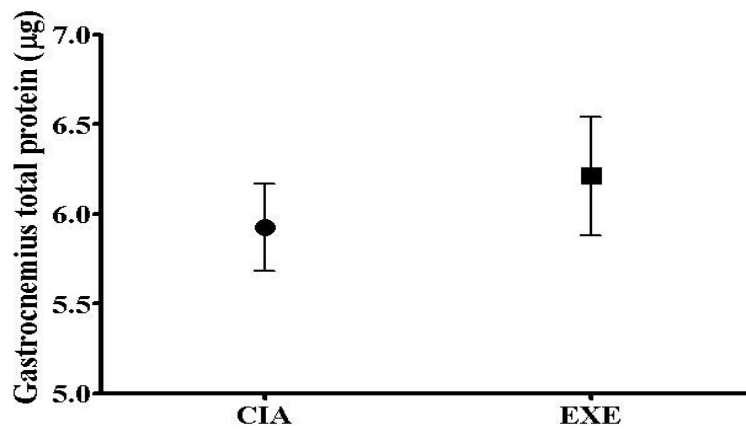


Fig6. Gastrocnemius muscles total proteins determined using Bradford assay. Symbols as in fig. 1. Each value represents the mean of normalizations \pm SEM; data were analyzed by Student's t test; $p < 0,05$.

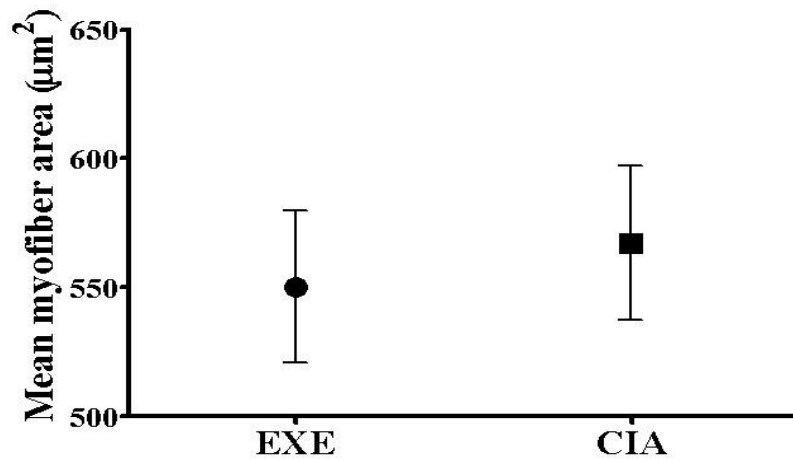


Fig7. Tibialis cross-sectional areas. Symbols as in fig. 1. Each value represents the mean of areas \pm SEM; data were analyzed by Student's t test; $p < 0,05$.

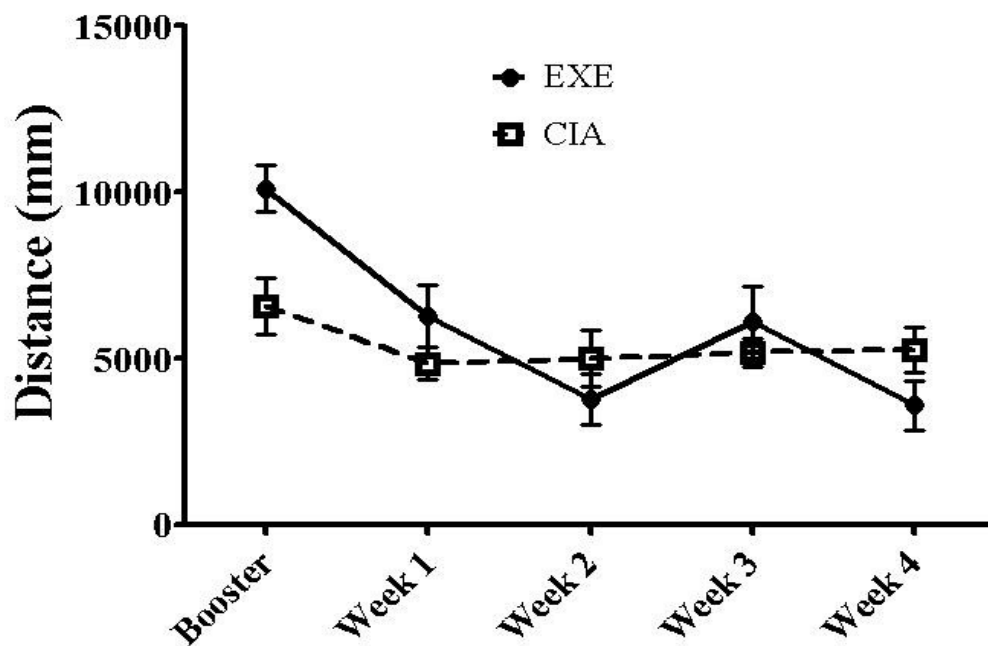


Fig8. Distances coursed in spontaneous locomotion. Symbols as in fig. 1. Each value represents the mean of distances \pm SEM; data were analyzed by Student's t test; $p < 0,05$.

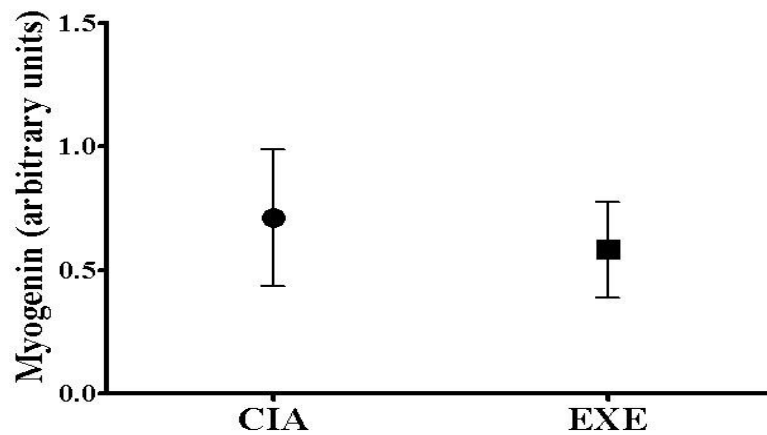


Fig9. Myogenin quantification. Expressions normalized to GAPDH. Each value represents the mean of normalizations \pm SEM; data were analyzed by Student's t test; $p < 0,05$.

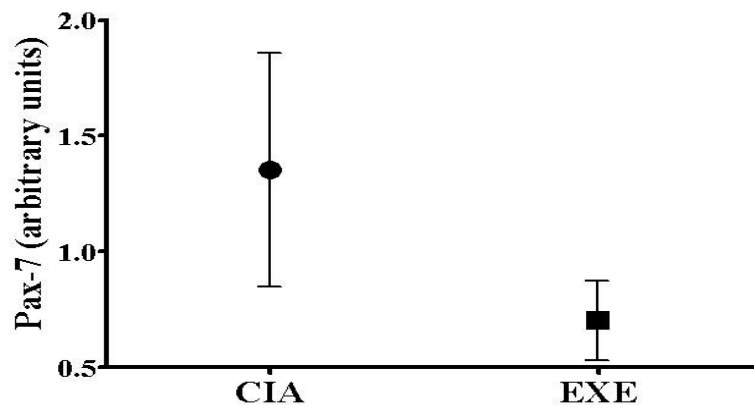


Fig10. Pax-7 quantification. Expressions normalized to GAPDH. Each value represents the mean of normalizations \pm SEM; data were analyzed by Student's t test; $p < 0,05$.

4 Discussion

Muscle wasting affects most RA patients, and its cause is assumed to be mostly related to pro-inflammatory cytokines, namely TNF- α and IL-1 β (6). Low physical activity and insulin resistance have been shown to play a role as well, whereas neither growth hormone deficiency (18) nor poor dietary intake (19) seem to be of concern as previously thought. Muscle wasting treatment has been challenging; drug therapies (e.g. TNF- α blockers (20,21)) and dietary supplements (22) might be possible countermeasures against cachexia, but neither of these approaches have shown evident effectiveness in reverting muscle loss. On the other hand, physical activities are assumed to play a major role in management of muscle wasting in RA. Indeed, exercise approaches has taken part in

arthritis care programs, since exercise is known to improve cardiorespiratory fitness, physical function (23–27) and muscle strength without disease exacerbation or additional joint damage (24,26,28–32). Some found through clinical trials that a combination of strength and endurance exercises over several weeks increased muscle mass and muscle strength (30). Similarly, high-intensity exercise interventions have shown to significantly improve muscle strength in RA groups in comparison to controls (26) or to low-intensity exercise groups (24,28).

There are no data in the literature regarding to role of exercise upon muscle wasting in animal models of arthritis. Previously, a pilot study carried out in our laboratory using a three times a week moderate exercise program upon male mice subjected to CIA resulted in significant increase in mean myofiber areas and no worsening of joint pathology (unpublished data). In experimental exercise approaches, choosing the type (aerobic vs resistance) and intensity (low, moderate or high) of the activities is a crucial step, in order to promote some degree of load upon muscle, which is essential to promote muscle regeneration; at the same time, excessive loads of exercise may result in additional joint damage. Some authors have verified the effects of different exercise loads upon joints in experimental models of osteoarthritis (33,34,35). Beckett et al. found significant increase in Mankin's score among animals subjected to excessive aerobic exercise during three and six weeks (33). In two studies of Galois et al., moderate exercise significantly lowered Mankin's score in comparison to controls (34) and even to low and high-intensity exercises (35). Taking this data into account, moderate exercise seems to be a safer approach.

In the present study, moderate aerobic exercise did not worsen clinical signs of joints such as arthritis severity, as shown by histological scoring, arthritis score and paw oedema, which reinforces that moderate exercise caused no further joint damage. Indeed, difference in paw odema ($p = 0,06$) almost reached a significant value, which might have been reached if we had used more animals. Our five-day exercise week rendered no significant improvement in muscle weight. This most likely led to an absence of significant improvement in body weights in the exercised group at the end of the study. We have not investigated whether the amount of food taken by the animals had influence upon weight parameters; weight loss in arthritic rats may be secondary (36) or not to reductions in food intake (37,38). In the same way, differences in myofiber areas were not of statistical significance between groups. Hence, the moderate exercise program used in our study was not able to revert loss of muscle mass in the CIA model.

Western blot analysis of myogenin and Pax-7 were carried out to verify the effect of exercise upon muscle proliferation and myogenesis. Myogenin is one of the myogenic regulatory factors, together with the products of *MyoD*, *Myf5* and *MRF4* genes; myogenin acts in the final phase of myogenesis by triggering the fusion of myocytes and the formation of myotubes (39). Pax-7 is a

transcription factor and a marker of satellite cells (39), and is commonly used to assess the total satellite cells in myofibers. The importance of Pax7-expressing satellite cells on skeletal muscle regeneration was demonstrated in a model using intramuscular diphtheria toxin, where muscle regeneration was achieved after transplantation of adult Pax7+ satellite cells (40). Our results show that exercise did not increase levels of the proteins analyzed in skeletal muscle of mice. Surprisingly, the mean levels of both factors were lower in the EXE group. These findings are not in agreement to studies showing increased satellite cells proliferation following exercise in healthy rodents (41–44). However, a relationship between inflammation and muscle regeneration has been stated by some authors. As pro-inflammatory cytokines, TNF- α (45) and IL-6 (46) mediate both muscle atrophy and regeneration. Castellero et al. observed increased levels of myogenic factor MyoD and myogenin in gastrocnemius muscle of arthritic rats in comparison to pair fed controls, although apparently these increased levels did not influence loss of gastrocnemius weight (38,36). From these data and the results of our immunoblots for Pax-7 and myogenin, we infer that an anti-inflammatory stimulus like exercise may result in decreased levels of myogenic factors. Nevertheless, further studies with larger samples are certainly necessary for this hypothesis to be confirmed.

Adjuvant-induced arthritis is an animal model of chronic inflammation, in which cachexia develops in a similar way to that on humans (47,48). This model has been a useful means of studying joint pathogenesis of RA in several studies, providing satisfactory results. By contrast, the effects of physical exercise upon muscles of animals subjected to CIA had not been put in practice until this time, according to our knowledge. Even though there are well established exercise programs for RA patients, it is difficult to propose a suitable experimental model of exercise that could resemble the ones that have successfully been applied to RA patients, namely with regard to exercise load and frequency. Novel exercise programs for experimental models need to be standardized, in order that the muscles to be exercised while the joints not to be damaged.

In our pilot study (mentioned above) with male mice only, exercise resulted in significant increase in mean myofiber areas; in the present study, female myofiber areas were not statistically different between exercise and sedentary groups. The fact of obtaining improvement only in male animals brings out the possibility of a role of androgens in muscle growth, highlighting the potential role of androgens in anabolic responses to exercise. It was already shown that testosterone induces muscle hypertrophy in humans (49–51) and increase in myofiber cross-sectional areas in male rats (52). Moreover, aerobic exercise was shown to increase testosterone levels in men (53), whereas increase in satellite cell number following exercise was seen only in young men (51). These data

could in part explain the lack of muscle improvements in the exercise-female mice used in our study.

Of note, one might consider the hard outcomes of CIA model upon pathological state of mice, seen that inflammation and joints edema develops in a way that even ankylosis of the limbs can develop. Owing to sore paws, mice sometimes tend to minimize the load upon the paws during training, which means that muscles might not be subjected to a suitable exercise load needed to revert muscle wasting. Moreover, sore paws are likely one of the features responsible for the lack of any increase in mice locomotion observed in our study. As observed by Hartog et al., locomotion and arthritis score are inversely correlated, and locomotion depends on muscle mass as well (12), and the same could be observed in a study carried out in our laboratory (55). Since there was no significant difference in muscle mass between groups, exercised mice probably had not been prone to course longer distances. For all these reasons, lowering the level of inflammation perhaps by administering an anti-inflammatory therapy (i.e. disease-modifying anti-rheumatic drug) concomitantly to exercise may provide a better clinical state to mice, so as to inflammation decreases and the exercises can be performed correctly. Furthermore, seeing that moderate exercise caused no damage to the joints, and high-intensity exercises have been suggested to improve muscle strength, an exercise program of higher intensity might be chosen in future studies in order to increase muscle mass in CIA model.

5 Conclusions

In this study, five-day exercise week did not have significant effects on myofiber areas, body weights, muscle weights and myogenesis transcriptional factors (myogenin and Pax-7), suggesting that this exercise program is not effective in reverting muscle loss in female CIA mice. These findings are indicative of a key role of androgens in muscle growth in this experimental model of arthritis. Further studies are needed to evaluate the effects of exercise on skeletal muscle and muscle wasting in CIA mice.

Conflict of interest The authors have declared that no conflicts of interests exist.

Disclosure statement All authors comply with the Ethical guidelines for authorship and publishing in the Journal of Cachexia, Sarcopenia and Muscle (47).

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