Original Research

Muscle wasting in collagen-induced arthritis and disuse atrophy

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Abstract

The mechanisms of muscle wasting and decreased mobility have a major functional effect in rheumatoid arthritis, but they have been poorly studied. The objective of our study is to describe muscular involvement and the pathways in an experimental model of arthritis compared to the pathways in disuse atrophy. Female Wistar rats were separated into three groups: control (CO), collagen-induced arthritis (CIA), and immobilized (IM). Spontaneous locomotion and weight were evaluated weekly. The gastrocnemius muscle was evaluated by histology and immunoblotting to measure the expression of myostatin (a negative regulator), LC3 (autophagy), MuRF-1 (proteasome-mediated proteolysis), MyoD, and myogenin (satellite-cell activation). The significance level was set at $P < 0.05$, and histological analysis of joints confirmed the severity of the arthropathy. There was a significant difference in spontaneous locomotion in the CIA group. Animal body weight, gastrocnemius muscle weight, and relative muscle weight decreased 20%, 30%, and 20%, respectively, in the CIA rats. Inflammatory infiltration and swelling were present in the gastrocnemius muscles of the CIA rats. The mean cross-sectional area was reduced by 30% in the CIA group and by 60% in the IM group. The expressions of myostatin and LC3 between the groups were similar. There was increased expression of MuRF-1 in the IM (1.9-fold) and CIA (3.1-fold) groups and of myogenin in the muscles of the CIA animals (1.7-fold), while MyoD expression was decreased in the IM (20%) rats. This study demonstrated that the development of experimental arthritis is associated with decreased mobility, body weight, and muscle loss. Both IM and CIA animal models presented muscle atrophy, but while proteolysis and the regeneration pathways were activated in the CIA model, there was no activation of regeneration in the IM model. We can assume that muscle atrophy in experimental arthritis is associated with the disease itself and not simply with decreased mobility.

Keywords: Muscle loss, experimental arthritis, locomotion, immobilization, regeneration

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Introduction

Chronic inflammatory diseases decreases body weight and induce muscle wasting.[1] Rheumatoid arthritis (RA) is an inflammatory systemic autoimmune disease with a prevalence of approximately 1% in the world population.[2] It is characterized by a chronic, symmetric, and erosive synovitis, predominantly of the peripheral joints, resulting in joint deformities and incapacitation. Muscle wasting is a common and important complication of RA, and it has been recently reported that low muscle mass is present in 20% of RA patients,[3] causing weight loss, decreased physical activity, and increased fatigue and weakness, which lead to a significant loss of functional capacity and quality of life with an elevated socioeconomic effect.[4,5]

The systemic mechanisms of muscle wasting are not fully elucidated, but most likely include anorexia, hormone dysfunction (insulin resistance and enhanced myostatin), and cytokine-driven hypermetabolism, particularly by tumor necrosis factor (TNF)-α.[6,7] Although experimental arthritis induces anorexia and results in no weight gain, it was demonstrated that muscle wasting was only partially due to the decrease in food intake.[8] Muscle wasting caused by RA has been associated with the intensity of inflammation and the severity of the disease.[9] In addition to RA, many other diseases that cause muscle wasting have been associated with higher inflammatory status, emphasizing the importance of inflammation in muscle loss.[7]

Muscle atrophy caused by disuse is a relatively less complex form of muscle loss and is typically dependent on the loss of mechanical input. This lack of stimuli triggers the activation of different proteolytic systems in muscles, leading to protein loss and muscle atrophy.[10]

Muscle loss is associated with skeletal muscle protein breakdown that can involve different proteolytic systems. These systems can be lysosomal proteases present in autophagy, proteases activated by calcium, such as calpain and caspase, and the ubiquitin-proteasome system.[11] Enzymes from the proteasome system specific to the skeletal muscle, MuRF-1 (muscle-specific RING finger protein 1) and atrogin-1/MAFbx (muscle atrophy F-box), have been investigated for their role in muscle atrophy.[12] In addition to protein degradation, deficient muscle satellite cell activation and muscle regeneration may be involved in muscle atrophy.[13] The participation of these mechanisms has not been well studied in RA or in experimental models of arthritis.

This study was designed to investigate the mechanisms involved in skeletal muscle atrophy in experimental arthritis and immobilization models, including histological analysis and investigation of the autophagy, proteasome proteolysis, and satellite cell activation. We demonstrate that both animal models developed muscle atrophy and that the muscle molecular markers analyzed did not have the same expression between the groups, suggesting the involvement of different intracellular pathways and indicating that muscle atrophy in arthritis occurs independently from the disuse caused by decreased activity.

Materials and methods

Animals and experimental groups

Female Wistar rats from 8 to 12 weeks of age were used. According to the protocol used, at this age, rats are susceptible to developing collagen-inducing arthritis.[14] The animals were reared at 20°C, with 12-h light–dark cycles and free access to food and water. All experiments were performed according to the Guiding Principles for Research Involving Animals (NAS) and to the Committee of Research and Ethics in Health and the Graduate Group of the Hospital de Clínicas de Porto Alegre.

The experimental animals were randomly divided into three groups: control (CO, $n = 5$–8), immobilized (IM, $n = 5$–8), and collagen-induced arthritis (CIA, $n = 5$–8). In the IM group, the left paws were immobilized with a copper boot, made from a copper sheet covered with a layer of cotton, for 10 days, the same time period as the period of disease in the arthritis animals (Figure 1).

Induction of arthritis

Arthritis was induced with bovine type II collagen (CII; Chondrex Inc., Redmond, WA, USA) dissolved in 0.1 M acetic acid at 4°C for 12 h and emulsified with complete Freund's adjuvant (CFA; Sigma, St Louis, MO, USA) containing inactivated *Mycobacterium tuberculosis*. Equal volumes of CII (2 mg/mL) and CFA (1 mg/mL) were mixed to form the emulsion.[14]

During the procedure, rats were anesthetized with isoflurane (1 mL/mL; Abbott Lab., Abbott Park, IL, USA). At day zero, 0.1 mL of emulsion was injected intradermically at the base of the tail to induce arthritis.[14] After 11 days, the rats received a reinforcement of the same emulsion in another site of the tail. Ten days after the booster injection, at day 21 after the first immunization, the animals were killed in a CO_2 gas chamber. At this stage, the animals had a score indicating severe arthritis, and a longer experimental time would expose them to great suffering. The gastrocnemius muscle, being very accessible and containing predominantly type II fibers, was dissected for histopathological analysis and stored at −80°C. The tibio-tarsic joint was removed to confirm the development of arthritis through histological analysis.

Assessment of locomotion

Before the induction of arthritis (day 0) and at the end of every week (days 7, 14, and 21), the rats were placed individually inside an acrylic 60 × 40 cm box (Monitor de Atividade IR, Insight Equipamentos Ltda® Ribeirão Preto, SP, Brazil). Spontaneous exploratory locomotion of the animals was detected by sensors bars located in the sides of the movement box for 5 min after 30 s of adaptation time (adapted from Ref. 15). The data from the movement detection were sent to and evaluated by the computer software Insight Equipamentos Ltda® (Ribeirão Preto, SP, Brazil) using the following parameters: route design, walked distance and velocity, and number of times standing and resting.

Figure 1 Images of the hindpaws of the different groups: a, control; b, immobilized; and c. collagen-induced arthritis (CIA). (A color version of this figure is available in the online journal)

Joint histopathology

The tibio-tarsic joints from the hind limbs were excised and fixed in 10% buffered formalin for five days. The joints were decalcified with 10% nitric acid for 24 h and paraffin mounted. The joints were sectioned into 4 μm slices and stained with hematoxylin-eosin (HE). The following score system was used to evaluate the individual joints and to measure the experimental arthritis severity by a blinded pathologist:[16] *synovial inflammation*—five high-powered 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*—0, absent; 1, mild (5–10 layers); 2, moderate (11–50 layers); 3, severe (>20 layers); *extension of pannus formation based on the reader's impression*—0, absent; 1, mild; 2, moderate; 3, severe; *synovial fibrosis*—0, absent; 1, mild (1–10%); 2, moderate (11–50%); 3, severe (51–100%); *cartilage erosion*, percentage of the cartilage surface that was eroded—0, absent; 1, mild (1–10%); 2, moderate (11–50%); 3, severe (51–100%); and *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).

Muscle histopathology

The gastrocnemius muscles from the hind limbs were excised and fixed in 10% buffered formalin for 1 day and paraffin mounted. The muscles were sectioned into 6 μm slices and stained with HE. The following score system was used by a blinded pathologist: *inflammatory infiltration*—0, absent; 1, mild (1–10%); 2, moderate (11–50%); 3, severe (51–100%); *edema*—0, absent; 1, mild (1–10%); 2, moderate (11–50%); 3, severe (51–100%); and *sarcoplasmatic rarefactions*—0, absent; 1, mild (1–10%); 2, moderate (11–50%); 3, severe (51–100%). Periodic acid Schiff (PAS) staining[17] was evaluated to determine if the myofibers with a higher concentration of glycogen were atrophic. The pathologist specifically looked into this preparation for the morphological changes that occur during apoptosis, including cells appearing as a round or oval mass with dark eosinophilic cytoplasm and dense purple nuclear chromatin fragments or apoptotic bodies consisting of cytoplasm with tightly packed organelles with or without a nuclear fragment.[18]

Immunohistochemistry

The slides were drained and incubated with anti-interleukin (IL)-1β (1:50 sc-1251; Santa Cruz Biotechnology, Dallas, Texas, USA) for 1 h at room temperature. The immunohistochemical staining was analyzed qualitatively by a blinded pathologist for the presence or absence of immunostaining.

Myofiber cross-sectional area

To determine the myofiber area, the muscle fiber diameter was measured, and the myofiber cross-sectional area (CSA) was calculated. Using the software Image-Pro Express (version 5.1.0.12; Media Cybernetics, Rockville, MD, USA), 10 images were taken of each gastrocnemius muscle per rat, and 20 fibers were measured from each image, totaling 200 measured myofibers.[19]

Immunoblot

For the western blot analysis, the muscles were homogenized with Tris buffer 10 mM and protease inhibitors. The sample protein concentration was measured using the Bradford assay. The membranes were marked with anti-myostatin (1:1000 sc-28310; Santa Cruz Biotechnology, Dallas, Texas, USA), anti-LC3 (1:1000 M5815; Sigma), anti-MuRF-1 (1:1000 sc-32920; Santa Cruz Biotechnology), anti-MyoD (1:1000 SAB4300397; Sigma), and anti-myogenin (1:1000 L8918; Sigma).[20] These protein expressions were normalized by β-actin expression.

Statistical analysis

The results are expressed as mean values with standard deviations (SD) for the symmetric variables and as medians and percentiles (25% and 75%) for the asymmetric variables. The data were compared by the Student *t* test, the χ^2 test and ANOVA, followed by Tukey's test. Significance was accepted at $P < 0.05$. The values were analyzed using the statistical package SPSS 13.0 (SPSS, Inc., Chicago, IL, USA).

Results

Animal locomotion

The spontaneous exploratory locomotion was evaluated weekly. In the last two weeks of the experimental period, the CIA animals walked less, walked with reduced velocity, stood up less, and rested more than the CO animals (Figure 2(b–e), respectively), which emphasizes the importance of an immobilization CO group to identify the atrophy factors not related to disuse.

Animal and muscle weight

The CO animals increased 20% in weight during the experimentation period, whereas the CIA animals maintained the same weight, although an increase in weight was observed at day 7. At the end of the experiment, the groups had a weight difference of 32% (Figure 2(a)). The gastrocnemius muscle weight and the relative muscle weight (muscle weight in mg divided by total animal weight in g) were lighter in the CIA group (0.89 ± 0.06 g and 5.06 ± 0.37 mg/g, $P < 0.01$) than in the CO group (1.30 ± 0.07 g and 6.35 ± 0.39 mg/g, $P < 0.01$), demonstrating significant loss of muscle tissue.

Joint histopathology

The histopathological study of the tibio-tarsic joints confirmed all of the parameters associated with the joint disease. The CIA animals presented synovial inflammation, hyperplasia, fibrosis, and cartilage and bone erosion, confirming the presence of severe arthritis (Supplementary Table 1). The joints of the IM animals had the same pattern as those of the CO group.

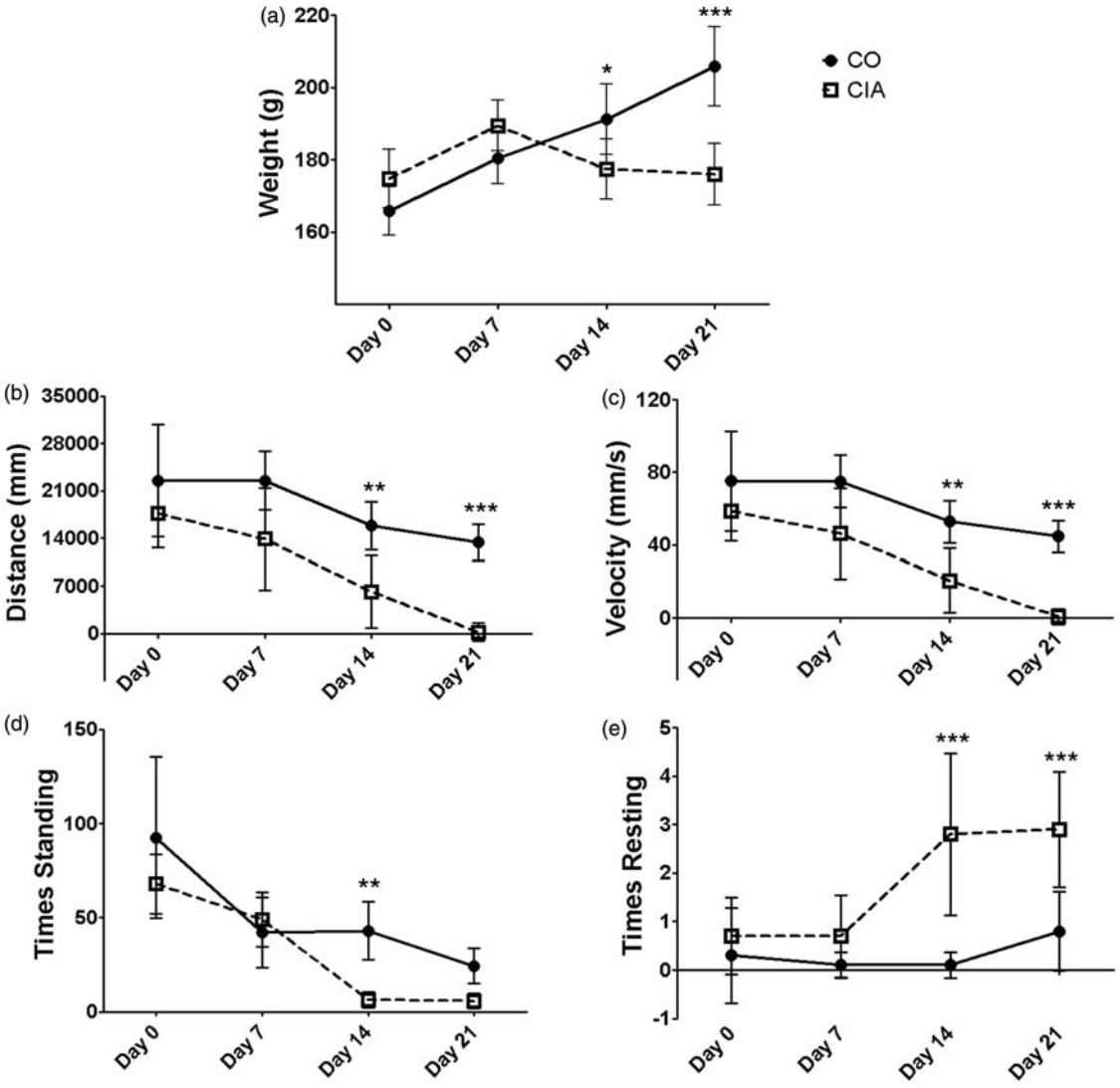

Figure 2 Weight (a), walking distance (b) and velocity (c), and times standing (d) and resting (e) of control (CO, •) and collagen-induced arthritis (CIA, □) animals. $^{*}P < 0.05$ versus control; $^{**}P < 0.01$ versus control; and $^{***}P < 0.001$ versus control. Data are shown as the mean ± SD.

Muscle histopathology

The gastrocnemius muscle sections stained with HE were evaluated. The muscles from the CIA animals showed inflammatory infiltration and edema, and the immune cells composing this inflammatory infiltration were mainly mononucleated cells. The muscles from the IM animals had myofiber edema without inflammation (Table 1). The morphological changes that occur during apoptosis, such as dense purple nuclear chromatin fragments or apoptotic bodies, were not observed in these preparations.

The myofiber CSA of the IM and CIA groups were significantly smaller than in the CO group, with a reduction of 34% and 60%, respectively (Table 1).

Although PAS is not the most accurate staining to differentiate myofiber types, it allows the observation of the glycogen quantity in myofibers. Typically, type 2 myofibers have higher levels of glycogen. The proportion of glycogen-poor and glycogen-rich fibers was similar in all experimental groups, although the myofibers that were more strongly stained (rich in glycogen) showed marked size reduction in the muscles of the CIA animals (Figure 3).

Immunohistochemistry

The immunohistochemistry for IL-1β demonstrated strong immunostaining of this cytokine in the muscles of the CIA animals, particularly where inflammatory infiltration was identified. IL-1β expression was marked only inside the blood vessels of the IM subjects and slightly in those of the CO group, and no immunostaining was identified in other parts of the muscle tissue (Figure 4).

Muscle molecular markers

After the histopathological study of the morphological changes, we focused on the molecular pathways involved in muscle loss. Through immunoblotting, the expression of myostatin (Figure 5), a negative regulator of muscle mass, and of LC3 (Figure 6), a marker of autophagy, remained

Table 1 Histological study and myofiber size of gastrocnemius muscles with hematoxylin-eosin staining of control (CO), immobilized (IM), and collagen-induced arthritis (CIA) groups

	CO ($n=7$)	IM ($n=5$)	CIA ($n=7$)
Inflammatory infiltrated			
Endomysium	0.1 ± 0.24	0.0 ± 0.0	$1.1 \pm 0.74^{**\#\#}$
Perimysium	0.0 ± 0.0	0.0 ± 0.0	$0.9 \pm 0.74^{**\#\#}$
Edema	0.1 ± 0.24	$1.75 \pm 0.5^{**}$	$0.8 \pm 0.79^{*\#}$
Regeneration	1.4 ± 0.7	1.7 ± 0.8	1.0 ± 0.0
Sarcoplasmatic vacuoles	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.32
Myofiber diameter (μm)	69.2 ± 9.57	$40.0 \pm 3.89^{***}$	$59.9 \pm 9.08^{**\#\#}$
Myofiber area (μm^2)	7175.6 ± 1418.2	$1396.3 \pm 121.3^{***}$	$4233.6 \pm 1066.1^{***\#\#}$

Values are expressed in mean $\pm$ SD of histological scores (scale 0–3 for inflammatory infiltrated, edema, and sarcoplasmatic vacuoles) of the ankles from rats and of myofiber size.
$^{*}P < 0.05$ versus CO group.
$^{**}P < 0.01$ versus CO group.
$^{***}P < 0.001$ versus CO group.
$^{\#}P < 0.05$ versus IM group.
$^{\#\#}P < 0.01$ versus IM group.

similar among the experimental groups ($P > 0.2$ and $P > 0.2$, respectively). Significantly increased expression of MURF-1 (indicative of proteosome activation) was observed in the IM (1.9-fold, $P < 0.01$) and CIA (3.1-fold, $P < 0.001$) groups compared to the CO group, with significant difference between the IM and CIA groups (1.6-fold, $P < 0.001$) (Figure 7). MyoD expression (a marker of satellite cell proliferation) was reduced in the IM group by approximately 20% ($P < 0.01$) (Figure 8) compared to the CO group, and myogenin (a marker of satellite cell differentiation) (Figure 9) expression was enhanced in the gastrocnemius muscle of the CIA rats (1.7-fold, $P < 0.001$) compared to that in the CO group.

Discussion

Although muscle atrophy is clinically common in RA[9] and has been described in experimental models,[21] little is known about its etiology and development mechanisms. Whereas disuse atrophy is a relatively uncomplicated form of muscle loss, dependent almost solely on the loss of mechanical input, in disease states associated with inflammation, such as cancer cachexia, AIDS, and sepsis, there is involvement of a complex procatabolic hormonal and cytokine environment.[10] Because of this clear difference in mechanisms and because inflammation and immobilization occur in RA, it is critical to study the muscle atrophy associated with arthritis in experimental models and to correlate the findings with those from models of disuse/immobilization.

During the development of arthritis in this study, the CIA rats gradually decreased their spontaneous locomotion. This could be especially observed at the end of the experimental observation; there was a 90% reduction of locomotion in the CIA animals (Figure 2(b)). Hartog *et al.*[15] observed impaired locomotion in CIA mice with more severe arthritis associated with muscle atrophy. This locomotion reduction could be interpreted as limbs disuse, which could lead to a synergistic effect with muscle wasting caused by inflammation.

At the end of the experimental procedure, the CO animals were 20% heavier than at baseline, whereas the CIA group maintained the same weight, although an increase in weight was observed at day 7 (Figure 2(a)). Although it was not quantified in this article, one important factor in weight change is food intake. Okiura *et al.*[21] and Filippin *et al.*[19] have described body reduction in mice with a CIA model, and Filippin *et al.*[19] described no decrease in the food consumption of these animals. In the inflammatory arthritis model of adjuvant arthritis, the animals maintained their initial weight and presented some degree of anorexia, observed as decreasing food intake.[8,22] In this same model, CO animals were given the same amount of food as arthritis animals consumed, and although they were lighter than *ad libitum* controls, they increased their weight in similar manner to the controls, demonstrating reduced weight is related but not dependent of food intake.[8,22]

With animal body weight, the gastrocnemius muscle weight of the CIA animals was reduced by 30%. Ozawa *et al.*[23] described a reduction of 20% in the muscle weight of rats with adjuvant arthritis. Hartog *et al.*[15] reported reduced weight of different types of muscles (soleus, gastrocnemius, tibialis anterior, and exterior digitorum longus) in a CIA mice model. Okiura *et al.*[21] have described body and muscle weight reduction in a CIA mice model, with a reduction in the relative muscle weight, confirming that there is accentuated muscle loss compared to total weight loss. In our study, the relative muscle weight of the CIA animals was 20% less than that of the CO animals.

Another method to evaluate muscle loss is through the size of the myofiber CSA, which is a measurement of muscle atrophy. The myofiber CSA was reduced by 34% in the CIA animals (Table 1), whereas Okiura *et al.*[21] observed a reduction of approximately 15% in the CSA of the myofibers of CIA animals, predominantly in those with severe arthritis. This difference in results is most likely due to the differences between the CIA model in rats and mice and the weight of these species because rats are 10× heavier. Da Silva[24]

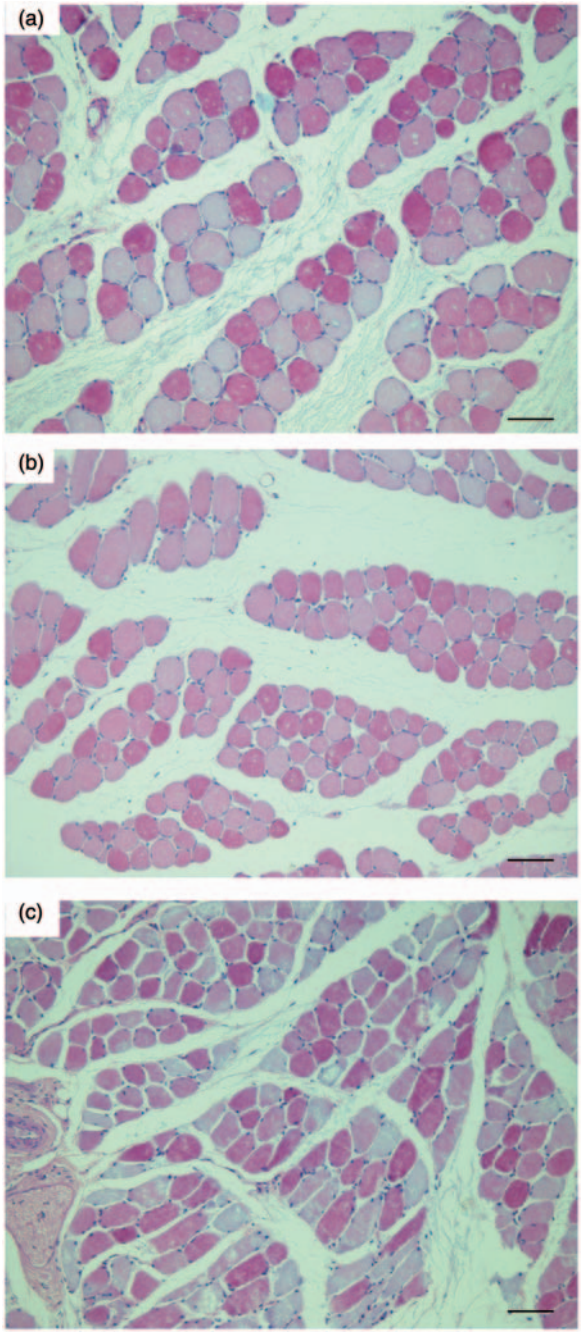

Figure 3 Images of the gastrocnemius muscle sections of the control (a), immobilized (b), and collagen-induced arthritis (c) animals stained with Periodic acid Schiff (amplification 400×). Scale bar (bottom right) = 50 μm. (A color version of this figure is available in the online journal)

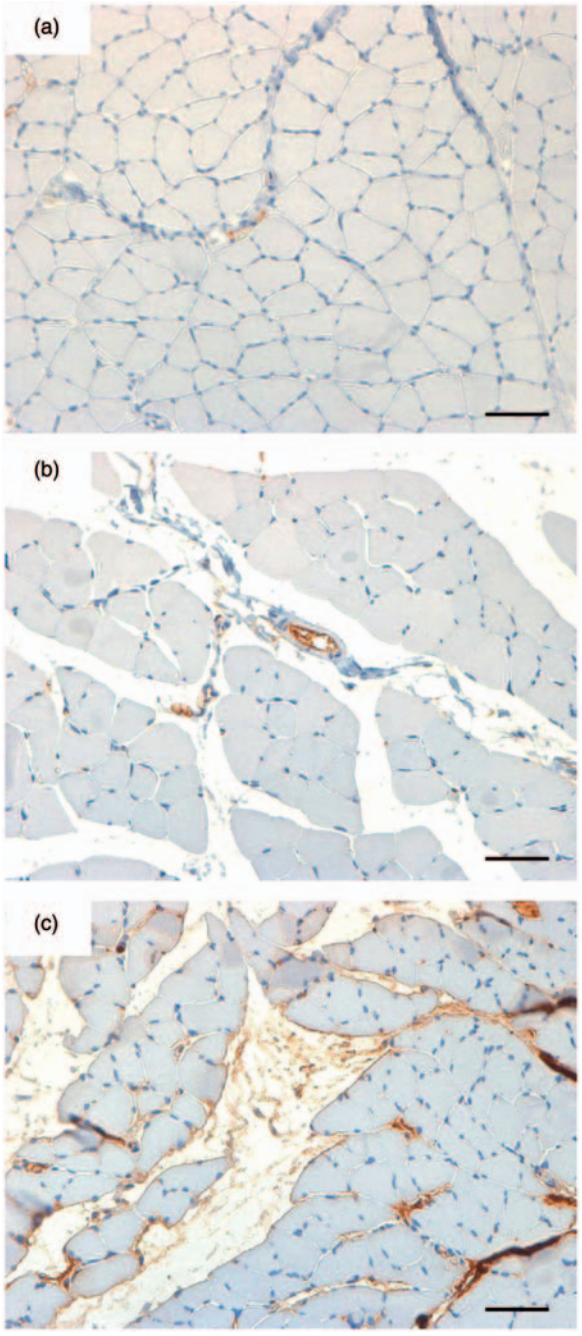

Figure 4 Immunohistochemistry for IL-1β of the gastrocnemius muscles of the control (a), immobilized (b), and collagen-induced arthritis (c) animals (amplification 400×). Scale bar (bottom right) = 50 μm. (A color version of this figure is available in the online journa)l

immobilized the hind limb paw of Wistar rats for seven days and detected a reduction of 43% in the myofiber CSA in the soleus muscle. In our study, there was a 60% reduction in the gastrocnemius muscles after 10 days of immobilization, confirming the efficacy of the immobilization model used.

In this study, edema and mild inflammatory infiltration were observed in the muscle histology of CIA mice, providing evidence for the presence of an inflammatory process (Table 1). Ozawa *et al.*,[23] using optical microscopy, did not identify pathological changes in the soleus and femoral muscles in a model of adjuvant arthritis. In the IM animals, there was edema without cellular infiltrate (Table 2), confirming the absence of significant inflammatory infiltration in the immobilized muscles. In addition to these visual signs of inflammation, immunohistochemistry showed a higher expression of IL-1β in the muscles of the CIA animals, whereas the IM group demonstrated no significant differences compared to the CO and CIA groups (Figure 4). This result agrees with our data on the inflammatory process in the muscles of the CIA animals. Pro-inflammatory

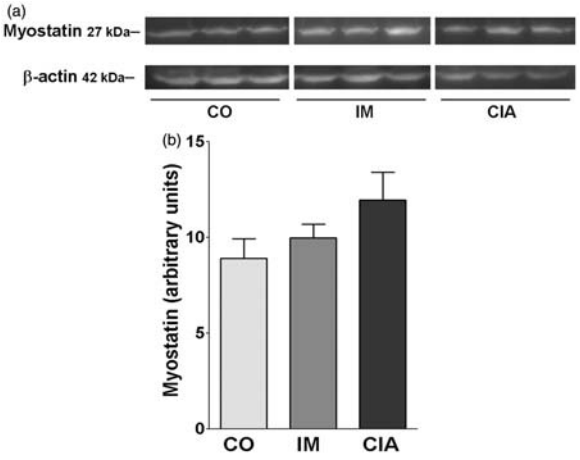

Figure 5 Myostatin expression by immunoblot analysis. (a) A representative image of the myostatin blot and the β-actin loading control. (b) Graph of the myostatin expression quantification of the control (CO), immobilized (IM), and collagen-induced arthritis (CIA) groups. Data are shown as the mean ± SD. Comparison among the groups was not significant

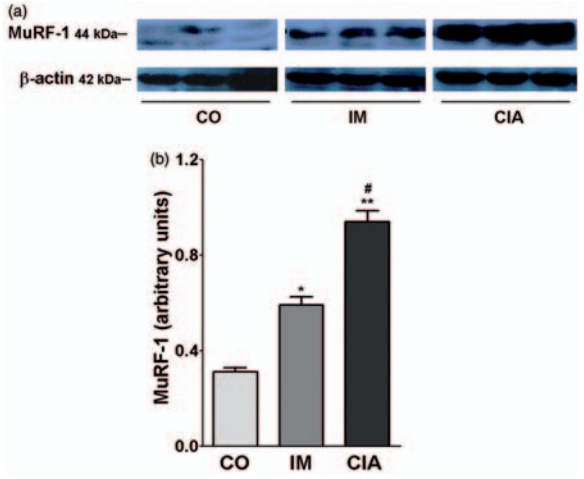

Figure 7 MuRF-1 expression by immunoblot analysis. (a) A representative image of the MuRF-1 blot and the loading control of β-actin. (b) Graph of the MuRF-1 expression quantification of the control (CO), immobilized (IM), and collagen-induced arthritis (CIA) groups. Data are shown as the mean ± SD. *$P < 0.01$ versus control; **$P < 0.001$ versus control; and #$P < 0.001$ versus immobilized. (A color version of this figure is available in the online journal)

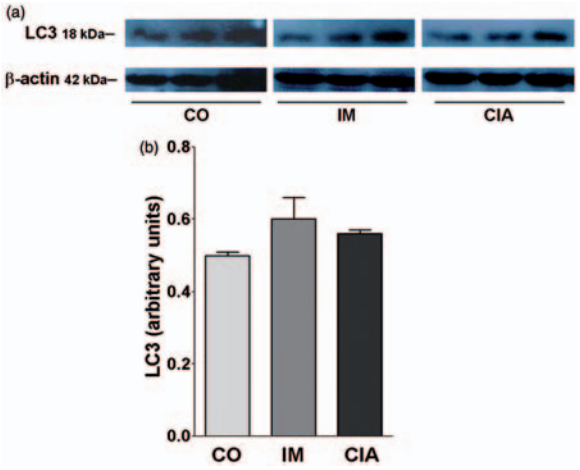

Figure 6 LC3 expression by immunoblot analysis. (a) A representative image of the LC3 blot and the loading control of β-actin. (b) Graph of the LC3 expression quantification of the control (CO), immobilized (IM) and collagen-induced arthritis (CIA) groups. Data are shown as the mean ± SD. Comparison among the groups was not significant. (A color version of this figure is available in the online journal)

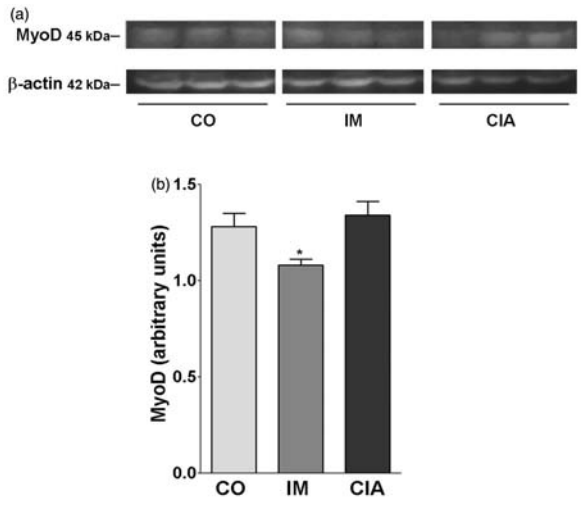

Figure 8 MyoD expression by immunoblot analysis. (a) A representative image of the MyoD blot and the loading control of β-actin. (b) Graph of the MyoD expression quantification of the control (CO), immobilized (IM), and collagen-induced arthritis (CIA) groups. Data are shown as the mean ± SD. *$P < 0.01$ versus control

cytokines, such as IL-1β and TNF, are directly related to RA pathogenesis. These cytokines stimulate weight[25] and muscle loss[26] and are involved in muscle regeneration mediated by satellite cells (SC).[27]

Muscle loss associated with arthritis could involve the entire muscle or primarily certain fiber types. The results in this study showed that atrophy was greater in the myofibers with higher glycogen levels (Figure 3). Most of the fibers richer in glycogen contain the type 2 myosin heavy chain,[28] but some type 1 fibers may also be in this group.[29] Although this subject is rarely studied, Castillero *et al.*[22] described a reduction in type 1 and type 2 myofibers in a rat model of adjuvant-induced arthritis, with a higher decrease in the fast twitch muscle fibers. Fink *et al.*[30] described fiber type 2 atrophy in patients with osteoarthritis who submitted to knee prosthesis surgery. In the literature, it is known that muscle atrophy first affects type II myofibers in aging and many diseases,[31] but the cause in not clear; the cause most likely involves an alteration in the key genes and proteins that define the myofiber type.[31]

Many mechanisms could lead to muscle loss, which could be related to altered production of several hormones and cytokines that induce negative systemic effects on muscle tissue. One of these signaling molecules is myostatin, a member of the transforming growth factor-β (TGF-β) superfamily that is a critical autocrine/paracrine inhibitor of skeletal muscle growth.[32,33] Myostatin expression is increased in prolonged food deprivation,[34] glucocorticoid-induced muscle atrophy[35] and skeletal muscle degeneration-related diseases such

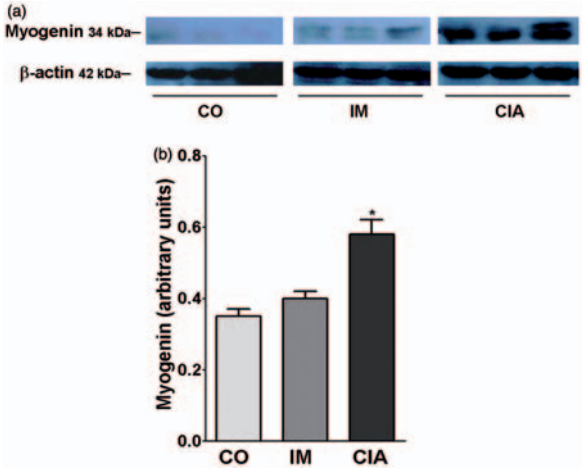

Figure 9 Myogenin expression by immunoblot analysis. (a) A representative image of the myogenin blot and the loading control of β-actin. (b) Graph of the myogenin expression quantification of the control (CO), immobilized (IM), and collagen-induced arthritis (CIA) groups. Data are shown as the mean ± SD. *$P < 0.001$ versus control. (A color version of this figure is available in the online journal)

as chronic illnesses[36] and other disorders.[37] Although increased in these processes, our results did not demonstrate changes in the expression of myostatin in the muscles of rats with CIA and IM (Figure 4). Corroborating our results, adjuvant-induced arthritis rat models have not identified differences in the expression of myostatin in the muscles.[8,22,38] On the other hand, in a mouse model of CIA, enhanced expression of myostatin was detected.[39] In disuse atrophy models, some articles found enhanced expression of myostatin.[40,41] This expression was only apparent until seven days, decreasing to values similar to those of the controls when exposed to a longer immobilization time.[41] Therefore, the expression of myostatin varies according to the disease type, animal model, and timing.

Another process involved in muscle loss is muscle proteolysis. The involvement of several proteolytic pathways has been described in muscle degradation, including autophagy, calcium-activated proteases (calpain and caspase), and the ubiquitin-proteasome system.[11]

Autophagy, an ancestor mechanism of cell survival by self-consumption of cytoplasmatic components during times of nutritional privation, has been demonstrated in the skeletal muscles of starving mice through the overexpression of LC3 (microtubule-associated protein 1 light chain 3)[42] but it has not been reported in inflammatory diseases. We did not observe activation of the autophagy pathway in either the CIA or immobilization models because LC3 expression did not differ from that of the CO animals (Figure 5).

One important system involved in muscle loss is the ubiquitin-proteasome pathway. Some studies have demonstrated that proteasome subunits and ubiquitin-ligase enzymes have enhanced expression during muscle atrophy, as well as increased expression of E3 ligases in different models of muscle wasting,[43] which shows the connection between E3 activity (MuRF-1 and atrogin-1) and loss of muscle mass.[43]

In this study, an enhanced expression of MuRF-1 was observed in the muscles of IM animals, which was even higher in CIA rats (3.1-fold) (Figure 6). Similarly, in studies with adjuvant induced arthritis, an experimental model of chronic articular disease, muscle increases of the E3 ubiquitin ligases MAFbx/atrogin-1, and MuRF-1 were observed in the adjacent gastrocnemius muscle,[8] as well as in other models of chronic inflammation.[44,45] A similar immobilization model detected higher expression of MuRF-1 (5.9-fold).[46] Increased expression of these ubiquitin ligases was also identified in other experimental models of disuse atrophy.[41,47] It appears that this ubiquitin ligase is increased in all forms of muscle atrophy.

Another possible mechanism of decreased muscle mass in arthritis could be deficient activation of SC, leading to decreased muscle regeneration. These cells may be activated in response to different growth, remodeling, and lesion stimuli, including inflammation.[20] MyoD and myogenin are used as molecular markers of SC and indicate different steps in the proliferation and differentiation of these quiescent myogenic precursors.[48] Our data demonstrated a decreased expression of MyoD in IM rats, while myogenin expression was increased in the muscles of the CIA animals and unchanged in the IM animals (Figures 7 and 8). The status of satellite cell activation in atrophy models is controversial in the literature. In similar models of disuse atrophy using a paw immobilization cast, MyoD and myogenin expressions were not increased.[49,50] There are studies showing reductions in the satellite cell myofiber area and nuclei number and a reduced expression of SC molecular markers (MyoD and myogenin).[51,52] Ramirez *et al.*[41] reported increased expression of these markers in a disuse atrophy model. They also detected a local inflammatory process with increased TNF-α muscle expression. Our disuse atrophy model did not present any signs of inflammation. It has been discussed in the literature that the expression of these proteins are triggered by the inflammatory process.[53] In an animal model of chronic inflammation (adjuvant arthritis), arthritic rats developed muscle atrophy with increased expression of MyoD and myogenin, linking proliferation and differentiation of SC to inflammatory conditions.[8] In an experimental model of muscular contusion causing an acute inflammatory response, our laboratory demonstrated the association of an inflammatory process and muscle regeneration involving the role of nitric oxide in the activation and differentiation of SC.[20]

It is important to point the possible effect of timing in the results. Whereas proteolysis is a constant stimulus in both inflammatory and disuse models,[41] SC should be stimulated at the beginning of inflammation. It is possible that at 10 days after the beginning of inflammation these cells had already passed by the processes of activation, proliferation, and initial differentiation (marked by MyoD) and were in the process of final differentiation and fusion with myofibers (marked by myogenin). This timing effect could explain the expression the markers in SC. MyoD remained similar to control while myogenin was increased in the CIA muscles. In the immobilization model, we did not find signs of inflammation, and thus no stimulus for regeneration,

hence the absent finding of satellite cell markers even in latter stages of satellite cell differentiation and fusion.

Besides SC activation and enhanced proteolysis, arthritis experimental models present decreased mobility (demonstrated in this manuscript, Figure 2) and pain,[54] insulin resistance,[55] and reduction in some hormones and growth factors as GH and IGF,[56] factors involved in muscle wasting as well. Based on these evidences, it seems that inflammation increases muscle proteolysis (3.1-fold of MuRF-1) and activates SC (1.7-fold of myogenin). However, this attempt to regenerate muscle is insufficient to compensate for the protein loss and to prevent muscle atrophy, since there is an unbalance between proteolysis and regeneration directed to protein loss.

This study demonstrated that the development of experimental arthritis is associated with decreased mobility, weight loss, muscle atrophy, and increased expression of markers of muscle proteolysis and regeneration, indicating that these processes may be involved in muscle loss caused by this disease. Proteosome-mediated proteolysis appears to be activated in IM animals and, to a greater degree, in CIA animals. While there was activation of the regeneration (MyoD and myogenin) pathway in the CIA group, that finding was not observed in the IM group. This difference in the activated pathways could explain the smaller myofiber CSA in the IM group. We can assume that muscle atrophy in experimental arthritis is associated with the disease itself and is not simply a result of decreased mobility because the IM group presented different pathway activation. Which pathways trigger muscle proteolysis and regeneration and their relationship with inflammation remain unclear.

Author contributions: VONT conceived the study design and coordinated the experimental procedures and the manuscript writing. LIF helped conceive the study, participated in its design, coordination, and experimental procedures, and helped draft the manuscript. PRV participated in all experimental procedures. PGO helped conceive the study and participated in the animal experimentation. RMX participated in the study design and coordination and helped draft the manuscript, critically revising it for important intellectual content. All the authors read and approved the final manuscript.

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