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Immobilization and therapeutic passive stretching generate thickening and increase the expression of laminin and dystrophin in skeletal muscle

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Abstract

Extracellular matrix and costamere proteins transmit the concentric, isometric, and eccentric forces produced by active muscle contraction. The expression of these proteins after application of passive tension stimuli to muscle remains unknown. This study investigated the expression of laminin and dystrophin in the soleus muscle of rats immobilized with the right ankle in plantar flexion for 10 days and subsequent remobilization, either by isolated free movement in a cage or associated with passive stretching for up to 10 days. The intensity of the macrophage response was also evaluated. One hundred and twenty-eight female Wistar rats were divided into 8 groups: free for 10 days; immobilized for 10 days; immobilized/free for 1, 3, or 10 days; or immobilized/stretched/free for 1, 3, or 10 days. After the experimental procedures, muscle tissue was processed for immunofluorescence (dystrophin/laminin/CD68) and Western blot analysis (dystrophin/laminin). Immobilization increased the expression of dystrophin and laminin but did not alter the number of macrophages in the muscle. In the stretched muscle groups, there was an increase in dystrophin and the number of macrophages after 3 days compared with the other groups; dystrophin showed a discontinuous labeling pattern, and laminin was found in the intracellular space. The amount of laminin was increased in the muscles treated by immobilization followed by free movement for 10 days. In the initial stages of postimmobilization (1 and 3 days), an exacerbated macrophage response and an increase of dystrophin suggested that the therapeutic stretching technique induced additional stress in the muscle fibers and costameres.

Key words: Immobilization; Muscle stretching; Soleus muscle; Laminin; Dystrophin; Mechanotransduction

Introduction

Mechanotransduction represents a form of intracellular signaling resulting from mechanical cell stress (1). In skeletal muscle, force is transmitted through a series of structures that are essential for maintaining the integrity of the muscle fibers (2) and regulating morphophysiological aspects of the cells (3,4).

In skeletal muscle fibers, the sarcolemma and the sarcoplasm are interconnected by a set of proteins that form the costameres. The dystrophin-glycoprotein complex is one of the costamere components. It stabilizes the sarcolemma and transmits the forces produced by or imposed on the muscle by the myofibrils to the extracellular matrix (ECM) and vice versa (5-7).

ECM proteins, such as laminin, bind to both membrane proteins and the dystrophin-glycoprotein complex (2). These associations interact with signaling proteins such as the phosphatidylinositol-3 kinase (8) in the process of protein synthesis.

Under conditions of hypokinesia, a decrease in contractile muscle activity contributes to the reduction of transduction forces that in turn generates alterations in contractile structures, non-contractile proteins, and intra- and extramuscular connective tissues (9,10). Decreased protein synthesis and increased proteolysis of muscle tissue can occur during chronic bed rest, periods of physical inactivity, or space flight (11).

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The reversibility of these alterations depends on the application of external stimuli, such as early mobilization (12) and passive stretching techniques (13-15). When a muscle is stretched, it transmits the force to a set of structures located in the extra- and intracellular milieu, such as laminin and dystrophin, respectively.

Tensile stimuli that exceed the plastic phase may cause micro-lesions and sarcolemmal damage, which can impair the integrity of dystrophin and the dystrophin-glycoprotein complex, resulting in an increase of permeability to ions and small molecules and lead to muscle degeneration and necrosis. Thus, previous studies have indicated that this costamere protein complex has a protective effect on the sarcolemma during force transmission (16). The chemical events that follow tissue injury may activate macrophages (17), and the number of macrophages in the injured area may reflect the magnitude of the inflammatory response.

Over the past 30 years, cross-sectional studies have analyzed the effects of stretching on muscles maintained in shortened immobilization; however, future studies should focus on the longitudinal axis, considering that this is where the tensile stretching forces are generated.

The involvement of dystrophin and laminin in force transmission is widely described in the literature (1,18,19). However, there are no reports about their expression after hypokinesia simulation and rehabilitation by passive stretching. It is possible that during immobilization, the total amount of cell proteins, including structural proteins such as laminin and dystrophin, could decrease since disuse induces proteolysis. Immunofluorescent staining may not detect these changes because the intra- and extracellular homeostasis should be preserved, especially by the function of dystrophin. Furthermore, external mechanical stimuli such as manual passive stretching could increase expression, and consequently, structural adaptations of these proteins would be detected using immunofluorescent staining.

In this study, we investigated how opposing longitudinal tensile forces and shortened immobilization, followed by remobilization by therapeutic passive stretching influenced the expression of laminin and dystrophin, which are representative proteins of the ECM and the costameres in the soleus muscle of female rats. The intensity of the macrophage response in tissue injury was also evaluated.

Material and Methods

Handling of animals and experimental procedures

One hundred and twenty-eight female Wistar rats weighing 250 g were divided into eight groups: immobilized (I), immobilized control [free for 10 days/control, $C_{(Immob)}$], immobilized and free for 1 day [$IF_{(1)}$], immobilized and free for 3 days [$IF_{(3)}$], immobilized and free for 10 days [$IF_{(10)}$], immobilized and stretched for 1 day [$IS_{(1)}$], immobilized and stretched for 3 days [$IS_{(3)}$], and immobilized and stretched for 10 days [$IS_{(10)}$]. The animals were kept four per plastic cage ($41 \times 34 \times 16$ cm) with free access to food pellets and water. This study was approved by the Ethics Committee for Animal Research of Faculdade de Medicina de Ribeirão Preto (No. 129/2009).

The immobilization model was previously proposed by Coutinho et al. (20) and Benedini-Elias et al. (21). The rats in groups I, $IF_{(1)}$, $IF_{(3)}$, $IF_{(10)}$, $IS_{(1)}$, $IS_{(3)}$, and $IS_{(10)}$ were anesthetized intraperitoneally with sodium thiopental (Thiopentax, Cristália, Brazil) and had their right hind limb immobilized in full plantar flexion to maintain the soleus muscle in a shortened position. The immobilization device was prepared with viscolycra fabric strips, cotton, No. 6 stainless steel mesh, micropore tape (3M Health Care, USA), adhesive tape (3M Health Care), impermeable surgical tape (Nexcare, 3M Health Care), silver tape (3M Health Care), and a stapler. The lower part, divided into anterior and posterior sections, consisted of stainless steel mesh with the margins wrapped with impermeable surgical tape. The anterior section was also wrapped and lined with cotton to protect the anterior surfaces of the immobilized limb and hip. The upper part was similar to a T-shirt made of viscolycra fabric. Next, the lower and upper portions of the device were joined with staples. The animals could freely move their head and forelimbs.

After immobilization for 10 days, the animals from group I were sacrificed by intraperitoneal injection of excess sodium thiopental (Thiopentax); the animals from groups $IF_{(1)}$, $IF_{(3)}$, and $IF_{(10)}$ were simply allowed to move freely in their cages for 1, 3, or 10 days, respectively. The animals from groups $IS_{(1)}$, $IS_{(3)}$, and $IS_{(10)}$ were subjected to a program of intermittent manual passive stretching with release in their cages between interventions for 1, 3, or 10 days, respectively. This stretching program comprised a daily series of 10 repetitions for 30 s each at 30-s intervals, measured with a chronometer. Manual passive stretching was performed by pushing on the plantar region in the dorsal flexion direction to stretch the soleus muscle of the right hindlimb (14,15,22).

At the end of the experimental procedures in each group, the animals were weighed and then sacrificed by an overdose of the sodium thiopental anesthetic. The soleus muscles of 8 animals in each group were processed for immunofluorescence analysis. The right hind limb of these rats was positioned in hip external rotation, knee extension, and tibiotarsal joint neutral position for measurement of the distance between the proximal and distal muscle-tendon junctions using a digital caliper (Marberg, Brazil). Then, the soleus muscle was excised and placed on Parafilm-covered (Pechiney Plastic Packaging Company, China) cork with the tendon ends fixed with pins. The original length of the muscle was maintained using the digital caliper. Following this process, the muscles were immersed in talcum powder and frozen in liquid nitrogen. The soleus muscles of 8 other animals in each group were

processed for Western blot (WB) analysis. The soleus muscle was excised, washed in sterile 0.9% saline, dried with filter paper, frozen in liquid nitrogen, and stored at –80°C until extraction of protein for WB.

Immunofluorescence analysis

Histological 5-μm thick longitudinal sections were obtained using a cryotome (Leica CM1850 UV, Leica Microsystems, Germany) (-25°C) and collected on glass slides (26 × 76 mm). Immunofluorescent staining was performed for laminin and dual-labeled for dystrophin and CD68, a protein located in the cell membrane of macrophages. Briefly, the slides were washed in phosphate-buffered saline (PBS) and fixed in Xpress molecular fixative (Sakura Finetek, Holland) for 4 min, washed three times in PBS for 5 min each, blocked with goat serum (Vector Laboratories, USA), diluted in 10% PBS for 60 min, blocked with avidin for 15 min (Avidin/Biotin Blocking Kit, Vector Laboratories), and washed briefly in PBS. Excess liquid was removed, and the samples were incubated with rabbit polyclonal anti-laminin primary antibody at 1:100 (Abcam, UK), or mixed with rabbit polyclonal anti-dystrophin at 1:400 (Abcam) plus mouse monoclonal [ED1] anti-CD68 at 1:200 (Abcam), or mixed with rabbit polyclonal anti-dystrophin at 1:400 (Abcam) plus mouse monoclonal [ED1] anti-CD68 at 1:200 (Abcam) at 37°C for 2 h. Following this, the slides were washed three times in PBS (5 min each) and incubated for 45 min with secondary antibodies (Invitrogen, USA) at 1:1000. Fluorescent goat anti-rabbit green 488 was the molecular probe for laminin; a mixture of fluorescent goat anti-rabbit green 488 plus goat anti-mouse red 568 was used for macrophage dystrophin. After three additional 5 min washes with PBS, the slides were mounted with Prolong Gold Antifade (Invitrogen). Morphometric analysis of the number of macrophages in histological sections was carried out using the Leica Application Suite (LAS) software (Leica LAS v 3.7); five random fields were captured using a Leica DM2500 microscope and DFC300FX digital camera (Leica Microsystems). A qualitative description of the spatial distribution of dystrophin and laminin in tissue sections was also obtained using a 40× objective lens (total field area 94,840.83 μm^2).

Western blot analysis

In all animals, the amounts of dystrophin and laminin were determined in homogenates of the lateral portion of the soleus muscle by immunoblotting 24 h after surgery. Fresh muscle was washed in cold PBS; tissue was removed and homogenized in extraction buffer and protease inhibitor cocktail (Sigma-Aldrich, USA). Total muscle protein (50 μg protein/well) was resolved by 5 or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes (Hybond-ECL; Amersham Pharmacia Biotech, UK). The membranes were blocked with 5% bovine serum albumin/Tris buffered saline with Tween 20 for 24 h and incubated overnight at 4°C with the primary rabbit polyclonal anti-dystrophin, 1:500 (Santa Cruz Biotechnology, USA) and rabbit polyclonal anti-laminin, 1:1000 (Abcam) antibodies. The blots were washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:10,000; Vector Laboratories) for 1 h at room temperature. The membranes were washed and developed using enhanced chemiluminescence (Amersham Pharmacia Biotech). Gel documentation was made using the Molecular Imager ChemiDoc XRS System (Bio-Rad, USA). Images were analyzed using the Image J program (developed at the National Institutes of Health and available at http://rbs.info.nih.gov/nih-image/), using the "Gel Analysis" functions. Results of the analysis included band values proportional to the integrated density and are reported in arbitrary units. Alpha-tubulin was used to determine approximately equivalent loading conditions.

Statistical analysis

The between-group analyses of the number of macrophages were carried out using a linear mixed-effect model. The model adjustment was performed using the SAS 9.2 software (SAS, USA). For the WB analysis, multiple comparisons were made using ANOVA, followed by the Bonferroni post-test. The 8 groups were compared using the Student *t*-test. The level of significance was set at 5% ($\alpha = 5\%$) and the confidence interval at 95%.

Results

The number of macrophages in the tissue was not changed by hypokinesia (Figure 1). The muscles of groups

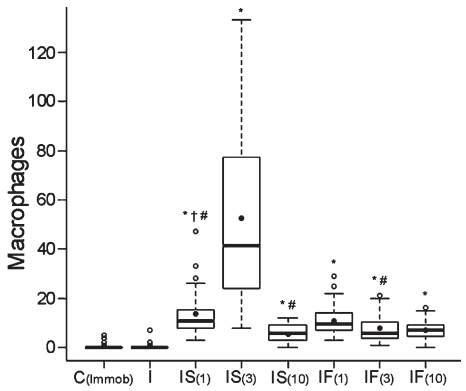

Figure 1. Number of macrophages in the soleus muscles. I: immobilized; $C_{(Immob)}$: immobilized control (free for 10 days/control); $IF_{(1)}$: immobilized and free for 1 day; $IF_{(3)}$: immobilized and free for 3 days; $IF_{(10)}$: immobilized and free for 10 days; $IS_{(1)}$: immobilized and stretched for 1 day; $IS_{(3)}$: immobilized and stretched for 3 days; $IS_{(10)}$: immobilized and stretched for 10 days. *$P<0.05$ compared to groups $C_{(Immob)}$ and I; †$P<0.05$ compared to $IS_{(10)}$; #$P<0.05$ compared to $IS_{(3)}$ (linear mixed-effect model).

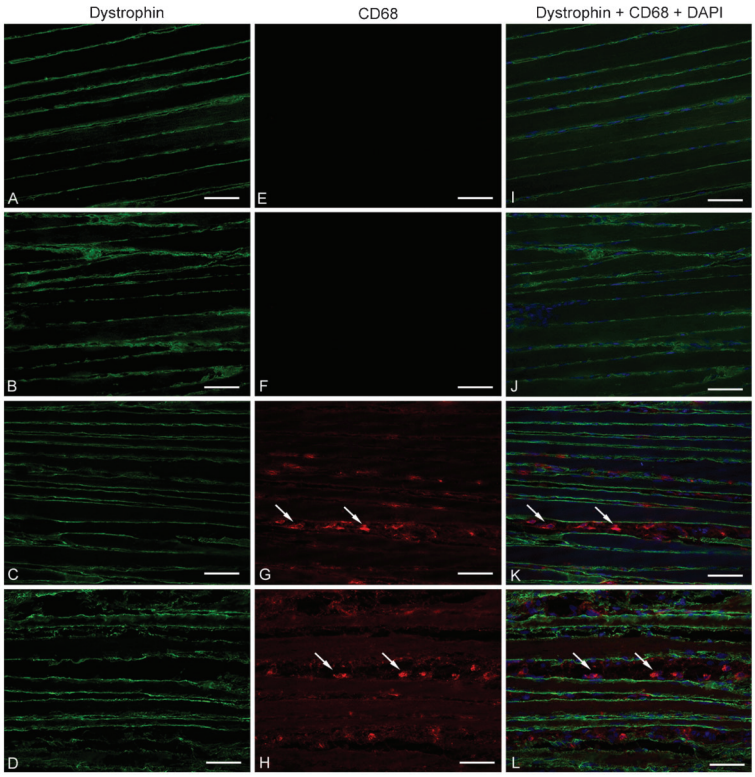

Figure 2. Photomicrographs of the soleus muscle of the animals in groups $C_{(Immob)}$, I, $IF_{(1)}$, and $IS_{(1)}$. *A-L*: Longitudinal sections immunolabeled with rabbit polyclonal anti-dystrophin (green fluorescence) and mouse monoclonal [ED1] anti-CD68 (red fluorescence) antibodies, nuclear fluorescent staining defined by DAPI (blue fluorescence). *A* [$C_{(Immob)}$]: Staining for dystrophin without changes. *B* (I): Staining thickness. *C* [$IF_{(1)}$] and *D* [$IS_{(1)}$]: Larger number of fibers in the image site, staining thickness, sarcolemmal undulations. *E* [$C_{(Immob)}$] and *F* (I): No staining of CD68 protein. *G* [$IF_{(1)}$] and H [$IS_{(1)}$]: Immunofluorescent staining of the CD68 protein expressed in the cellular membrane of macrophages (arrows). *I* [$C_{(Immob)}$] and *J* (I): Merged images of dystrophin and DAPI. *K* [$IF_{(1)}$] and *L* [$IS_{(1)}$]: Merged images of dystrophin, CD68, and DAPI. Observe the location of macrophages and their respective nuclei (arrows). See Figure 1 legend for explanation of abbreviations. Scale bars: 50 μm.

$IF_{(1)}$, $IS_{(1)}$, $IF_{(3)}$, $IS_{(3)}$, $IF_{(10)}$, and $IS_{(10)}$ had increased numbers of macrophages compared with $C_{(Immob)}$ and I ($P<0.05$; Figures 2G and H, and 3E-H). The isolated freedom of movement in cages did not result in significant differences between groups $IF_{(1)}$, $IF_{(3)}$, and $IF_{(10)}$. On the other hand, the animals subjected to stretching for 3 days (IS_3) had significantly more macrophages than $IF_{(3)}$, $IS_{(1)}$, and $IS_{(10)}$ ($P<0.05$; Figures 1 and 3F).

The spatial distribution of dystrophin along the longitudinal axis of the soleus muscle fibers is shown in Figures 2 and 3 (A-D). The soleus muscle of group I showed fluorescence thickening for dystrophin when compared to $C_{(Immob)}$ (Figure 2B and A, respectively). The soleus muscle in groups $IF_{(1)}$ (Figure 2C) and $IS_{(1)}$ (Figure 2D) had more fibers in the image area, fluorescence thickening for dystrophin, and undulations of the sarcolemma, mainly in group $IS_{(1)}$. In groups $IF_{(3)}$ (Figure 3A) and $IS_{(3)}$ (Figure 3B), some fibers expressed discontinuous dystrophin staining along their length, which suggests breaks within the sarcolemmal membrane. Both sarcolemmal undulations and sparse dystrophin expression were observed in areas with tissue loss. Fluorescence thickening for dystrophin was also observed in both groups $IF_{(10)}$ (Figure 3C) and $IS_{(10)}$ (Figure 3D), but was more evident in the former.

The quantitative WB analysis of dystrophin content in the soleus muscle indicated that immobilization increased the amount of this protein compared with $C_{(Immob)}$ [$C_{(Immob)}$ *vs* I, $P<0.05$; Figure 4A]. A similar increase was observed in $IS_{(3)}$ [$C_{(Immob)}$ *vs* $IS_{(3)}$, $P<0.05$]. Furthermore, among the groups subjected to stretching, $IS_{(3)}$ had the largest amount of dystrophin. Nevertheless, the amount of this protein decreased significantly in groups $IS_{(1)}$, $IS_{(10)}$, $IF_{(1)}$, $IF_{(3)}$, and $IF_{(10)}$ compared with the immobilization group ($P<0.05$), and they also had values similar to the control group ($P>0.05$; Figure 4A).

The spatial distribution of laminin along the longitudinal axis of the soleus muscle fibers is shown in Figures 5 and 6 (A-D). Discrete thickening of laminin fluorescence was more frequent in Group I than in $C_{(Immob)}$ (Figure 5B and A, respectively). Figures 5C,D and 6C,D show a higher number of fibers and fluorescence thickening for laminin in the image area (groups $IF_{(1)}$, $IS_{(1)}$, $IF_{(10)}$, and $IS_{(10)}$, respectively). Figure 6A and B show laminin expression in the intracellular milieu in groups $IF_{(3)}$ and $IS_{(3)}$.

The quantitative WB analysis in the soleus muscle indicated an increased amount of laminin in the immobilized animals compared with the values obtained in control animals ($P<0.05$; Figure 4B). Compared with immobilization, stretching and permitting free movement in the cage for 1, 3, and 10 days did not significantly change the amount of this protein ($P>0.05$; Figure 4B). However, after 10 days of free movement in the cage [$IF_{(10)}$], the values were higher than those obtained in group $C_{(Immob)}$ ($P<0.05$; Figure 4B).

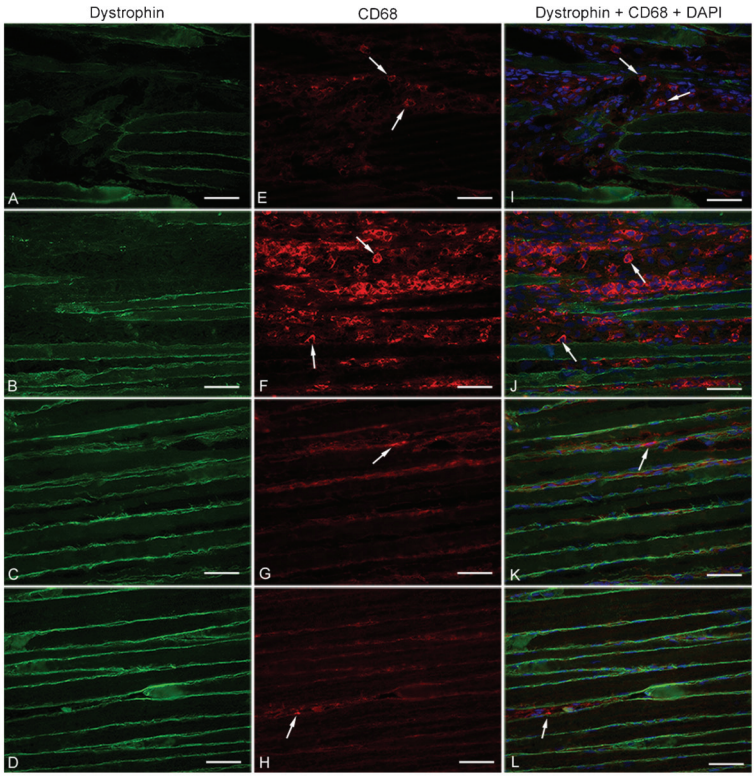

Figure 3. Photomicrographs of the soleus muscle of the animals in groups $IF_{(3)}$, $IS_{(3)}$, $IF_{(10)}$, and $IS_{(10)}$, *A-L*: Longitudinal sections immunolabeled with rabbit polyclonal anti-dystrophin (green fluorescence) and mouse monoclonal [ED1] anti-CD68 (red fluorescence) antibodies, nuclear fluorescent staining defined by DAPI (blue fluorescence). *A* [$IF_{(3)}$] and *B* [$IS_{(3)}$]: Staining discontinuity, sarcolemmal undulations and sparse staining for dystrophins. *C* [$IF_{(10)}$] and *D* [$IS_{(10)}$]: Staining thickness and some sarcolemmal undulations. *E* [$IF_{(3)}$], *F* [$IS_{(3)}$], *G* [$IF_{(10)}$], and *H* [$IS_{(10)}$]: Immunofluorescent staining of the CD68 protein expressed in the cellular membrane of macrophages (arrows). *I* [$IF_{(3)}$], *J* [$IS_{(3)}$], *K* [$IF_{(10)}$], and *L* [$IS_{(10)}$]: Merged images of dystrophin, CD68, and DAPI. Observe the location of macrophages and their respective nuclei (arrows). See Figure 1 legend for explanation of abbreviations. Scale bars: 50 μm.

Discussion

This study analyzed changes in the spatial distribution and amounts of laminin and dystrophin expressed in the soleus muscle in animals immobilized for 10 days, after remobilization via isolated free movement, or associated with intermittent manual passive stretching for up to 10 days.

Laminin is an ECM structural protein responsible for force transmission to the sarcolemma and vice versa (2). This protein binds to dystrophin through the α-dystroglycan (23). Dystrophin is present along the sarcolemma, at the muscle-tendon junction, and at the intrafascicular termination, suggesting its potential involvement in transmitting tension from the muscle fiber to neighboring fibers, either in series or in parallel, via the ECM (24). The dystroglycans are adhesion molecules of skeletal muscle (1,9) that transmit force to dystrophin and laminin (1). The results of this study showed that 10 days of hypokinesia by

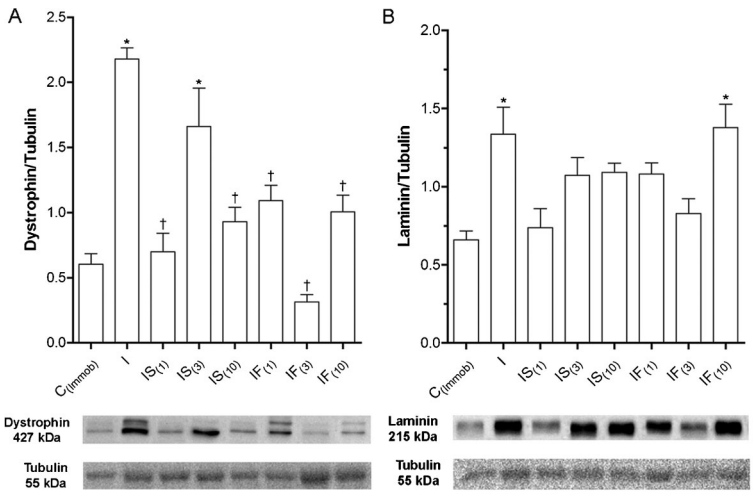

Figure 4. Western blot analysis for dystrophin (*A*) and laminin (*B*) in the soleus muscle of animals in the groups analyzed. See Figure 1 legend for explanation of abbreviations. *$P<0.05$ compared to $C_{(Immob)}$; †$P<0.05$ compared to I (Student *t*-test).

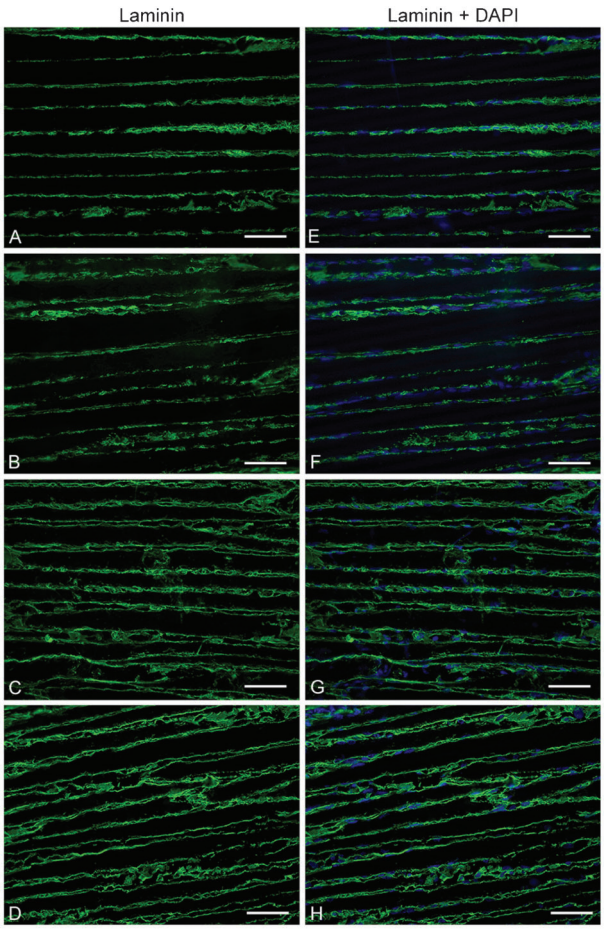

Figure 5. Photomicrographs of the soleus muscle of the animals in groups $C_{(Immob)}$, I, $IF_{(1)}$, and $IS_{(1)}$. *A-H*: Longitudinal sections immunolabeled with rabbit polyclonal anti-laminin (green fluorescence) antibody, nuclear fluorescent staining defined by DAPI (blue fluorescence). *A* [$C_{(Immob)}$]: Staining for laminin without alterations. *B* (I): Staining thickness. *C* [$IF_{(1)}$) and *D* [$IS_{(1)}$]: Larger number of fibers in the area and staining thickness. *E* [$C_{(Immob)}$], *F* (I), *G* [$F_{(1)}$], and *H* [$IS_{(1)}$]: Merged images of laminin and DAPI. Observe the localization of the nuclei (in blue). See Figure 1 legend for explanation of abbreviations. Scale bars: 50 μm.

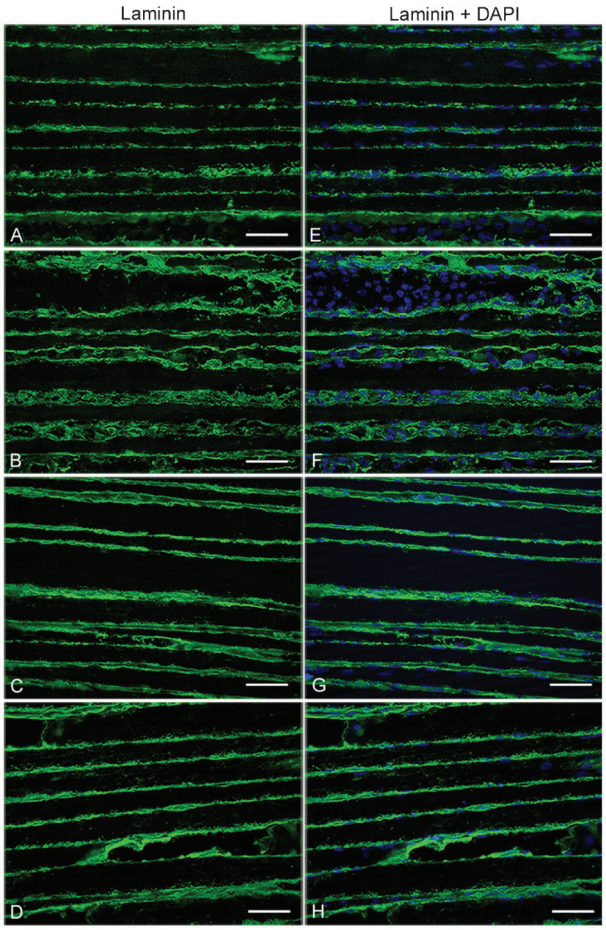

Figure 6. Photomicrographs of the soleus muscle of the animals in groups $IF_{(3)}$, $IS_{(3)}$, $IF_{(10)}$, and $IS_{(10)}$. *A-H*: Longitudinal sections immunolabeled with rabbit polyclonal anti-laminin (green fluorescence) antibody, nuclear fluorescent staining defined by DAPI (blue fluorescence). *A* [$IF_{(3)}$] and *B* [$IS_{(3)}$]: Staining thickness and laminin expression in intracellular areas. *C* [$IF_{(10)}$] and *D* [$IS_{(10)}$]: Staining thickness. *E* [$IF_{(3)}$], *F* [$IS_{(3)}$], *G* [$IF_{(10)}$], and *H* [$IS_{(10)}$]: Merged images of laminin and DAPI. Observe the localization of the nuclei (in blue). See Figure 1 legend for explanation of abbreviations. Scale bars: 50 μm.

immobilization increased the amount of dystrophin and laminin in the soleus muscle. It also indicated that there were no areas of dystrophin breakdown in the sarcolemma. However, Powers et al. (11) suggested that muscle inactivity leads to a reduction of signal transduction in muscle fibers, with a consequent reduction of protein synthesis and increased proteolysis. In contrast, Anastasi et al. (25) analyzed the expression of costamere proteins (sarcoglycan and dystrophin) in the gastrocnemius muscle of patients with polyneuropathy, which is a form of physical inactivity, and observed a normal distribution of dystrophin associated with reduced sarcoglycan staining. Although there is no consensual explanation for these findings, our results indicate that the costamere proteins responded positively to the low functional demand on the soleus muscle during 10 days of hypokinesia.

Our cytoarchitectural analysis of myofibers that were immobilized and then remobilized with free movement in the cage or with therapeutic stretching showed a discontinuous pattern of dystrophin labeling and sarcolemma undulations, mainly when passive stretching was performed for three days. Existing literature indicates that passive stretching, and to a lesser extent, isolated free movement, may result in the discontinuity of ECM or of sarcolemma components (26-28). Despite the discontinuous appearance of sarcolemma identified by histopathological analysis, WB revealed a

significant increase in the amount of dystrophin in group $IS_{(3)}$ compared with the control group. In a clinical study, Kosek and Bamman (7) observed an increase in the amount of dystrophin in the human vastus lateralis muscle 24 h after an initial resistance-training session. In contrast, Lovering and De Deyne (29) observed a significant reduction of dystrophin expression through WB analysis, 3 days after the application of a single eccentric stimulus in Sprague-Dawley rats. These conflicting results suggest that the tissue response not only depends on the intensity and/or the frequency of the stimulus application, but also on the type of muscular activity (active or passive). The histopathological analysis showed that the thickening of the laminin-staining pattern in the intracellular milieu was more evident in $IS_{(3)}$ than in group $IF_{(3)}$. The presence of intracellular laminin was also reported by Smith et al. (30) 48 h after eccentric contractions in the gastrocnemius muscle of female rats as indicative of tissue injury.

Laminin is an ECM glycoprotein composed of three different polypeptide chains (31). It binds to the dystrophin-glycoprotein complex that interacts with both contractile proteins and signaling proteins for protein synthesis such as phosphatidylinositol 3-kinase (1,8,32). De Deyne (18) reported that the transmission of passive force is exerted initially on collagen, followed by glycoproteins and membrane proteins such as integrins. Sequentially, this transmission of passive force promotes the activation of the cytoskeleton bound to costamere proteins, followed by the non-contractile cytoskeleton array, and ultimately by the contractile proteins. Such associations act like mechanoreceptors when subjected to a mechanical stimulus, as they transmit energy and transform it into biological events (1,19). In this study, a thickening of both proteins was observed throughout the experimental period, postimmobilization procedure, and recharge period. These findings suggest that the transmission of positive and negative forces mutually influenced the proteins, resulting in changes in their respective spatial conformations.

Analysis of the WB results showed that the immobilization procedure significantly increased the expression of these proteins, which corroborates the aforementioned findings. Significant increases were also observed for dystrophin in group $IS_{(3)}$ and laminin in group $IF_{(10)}$ compared with the values obtained in group $C_{(Immob)}$. Comparison of the results obtained in the other groups did not show other statistically significant differences.

The number of activated macrophages found in the soleus muscle of the animals in group I was similar to that found in animals of group $C_{(Immob)}$. However, 1 day of recharge, with or without stretching, resulted in an increased number of macrophages, suggesting that there was a tissue injury (17). McLennan (33) observed $ED1^+$ macrophages in the peripheral portion of the anterior tibialis muscle of Wistar rats 3 h after application of cryolesions. Our results, obtained after 3 days of remobilization, suggested an intensification of injury, as areas with a loss of functional tissue were visualized and there was a considerable increase in number of macrophages, mainly in group $IS_{(3)}$. Some macrophages were still present after 10 days of remobilization. According to McLennan (33) and Järvinen et al. (5), macrophages release chemotactic signals such as cytokines and growth factors that trigger other circulating inflammatory cells, intensifying the inflammatory response. The maintenance of overload stimuli throughout the experimental period may have exacerbated the inflammatory response in the postimmobilization phase as well. Furthermore, the present study could not establish the isolated effect of manual passive stretching. After the procedure, animals remained free in the cage for about 23 h, which also resulted in a type of longitudinal stress to tissues. Also, some serum variables and inflammatory markers could increase data regarding the intensity of tissue damage and the recovery induced by the procedures discussed here.

In conclusion, the expression of costamere proteins seemed to adjust positively to low demand being placed on the soleus muscle during 10 days of hypokinesia, and such positive adjustment was maintained during the remobilization period, particularly when associated with intermittent stretching.

Further studies should be carried out to determine the mechanical significance of these findings and their impact on the transmission of passive and active forces on other elements of costameres (including glycoproteins) and the ECM (including integrins). The discontinuity of these elements observed along the sarcolemma suggests the interstitial vulnerability of the extracellular compartment of these structural elements, which is reinforced by the appearance of a large number of macrophages at an early stage (3 days) of postimmobilization.

Clinical implications

Immobilization of muscles in a shortened position is a frequently used medical treatment. In clinical rehabilitation, stretching exercise is the therapeutic intervention most often indicated when the range of motion is reduced and functional limitations are found. The present results indicated that disuse and stretching induce alterations in structural proteins. Although these results show injury induced by the stretching, it does not contraindicate its clinical application because the treatment objective, which was the increase in muscle length and tissue integrity, was reached.

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