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From Cell Biology to Tissue Engineering

Effect of rehabilitation protocols on muscle function and morphology following hindlimb disuse in weanling rats

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Summary. Background: Primary or secondary disorders in developing skeletal muscles are prevalent in physical therapy practice. Assessment of gait functional changes and morphological aspects of hindlimb muscles of weanling rats have not been reported simultaneously in the literature. Rehabilitation by active (eccentric training) and passive (stretching) exercises after hypomobility needs to be investigated. Methods: After ten days of immobilisation in a plantar flexion-shortened position, animals underwent eccentric training on treadmills, intermittent (a single series of ten exercises of 30 seconds each, with a 30-s interval) or continuous stretching protocols for 40 minutes, or had free cage activity for three days. Analysis of gait variables and muscle morphology (immunohistochemical staining of soleus and plantar muscles for fibronectin and types I and III collagen and immunofluorescence staining for dystrophin, laminin, Pax-7, and CD68) were performed. Results: On the third day, the rehabilitated animals touched the ground surface with their toes, except for the group undergoing continuous stretching. The total amount of extracellular macrophages was higher in the rehabilitated animals. The number of satellite cells was not significantly different between groups. Conclusion: Three days of active training (eccentric exercise) showed greater effectiveness compared to the other rehabilitation programs. Weanling rats seem to respond differently to external stimuli such as disuse and remobilisation.

Key words: Collagen, Fibronectin, Laminin, Dystrophin, Pax-7, CD68

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Introduction

During childhood, skeletal muscle undergoes growth adaptations to prepare for adult life. Postnatal skeletal muscle diseases are clinically prevalent; however their effects on rehabilitation and morphological changes have been minimally explored.

Reduced physical activity, involving conditions such as a bedridden situation or immobilisation, causes decreased functional excursion and reduced force production of the involved muscles (Okita et al., 2004). Young and developing muscles show greater adaptations to decreased muscular activity compared to adults (Goldspink et al., 1992).

Muscular alterations caused by disuse are reversible, and this reversibility is positively influenced by the introduction of external stimuli, such as early mobilisation (Hwang et al., 2006), stretching techniques (Gomes et al., 2007) and eccentric training (Sakakima et al., 2004; Cornachione et al., 2008). Muscular stretching is an appropriate therapy when range of motion limited the function. Stretching is a technique capable of inducing myofibrillogenesis, by lengthening longitudinally the costameric structures, intermediate filaments, contractile proteins and extracellular matrix elements (Coutinho et al., 2004). However, when the

tissue undergoes prolonged stretch beyond its plastic phase, significant cellular degeneration appears as ultrastructural alterations (Mattiello-Sverzut et al., 2006; Gomes et al., 2007). Eccentric exercises have also been shown to improve recovery of disuse-induced morphofunctional alterations (Brasileiro et al., 2011; Cornachione et al., 2011). Following muscle disuse, female rat weanlings were benefitted from 10 and 21 days of eccentric training; muscle analysis verified greater capillarity, tissue reactivity, and nuclear centralisation at 10 days.

Stretching and eccentric exercise initiate passive and active longitudinal tensile loads, respectively, in the muscle. With passive stretching, it is assumed this induces structural adjustments by initiating signal transduction in the non-contractile elements, from the extracellular into the intracellular compartment. On the other hand, eccentric contraction induces an active stimulus starting from the contractile elements of the muscle fibres and secondarily affecting the non-contractile elements of the intracellular and extracellular compartments. In this context, the factors involved in the cellular adaptation need to be explored and clarified in muscles of weanling and adult animals. Recently, Sun et al. (Sun et al., 2013) identified that the expression of NOS1, an activity-dependent marker, is reduced in the soleus muscles of adult animals undergoing passive movement after disuse, but it was not affected with active movement. Expression of the costameric elements laminin and dystrophin increased in solei after three days of remobilisation with intermittent stretching following 10 days of immobilisation in a shortened position (Cação-Benedini et al., 2014).

Muscle fibre growth can involve satellite cells, myogenic stem cells whose function is intimately linked to regeneration and muscular repair. These cells express myogenic markers such as M-cadherin, Pax-7, Myf5, and NCAM/CD56 and present the capacity for self-renewal. Furthermore, they are quiescent in skeletal muscle and are activated by molecules, whereupon they proliferate and differentiate to begin muscular regeneration (Christov et al., 2007). The absolute number of satellite cells increases with age and shows similar percentages between young and adult rats (Brooks et al., 2009). In addition, skeletal muscle satellite cell content was enhanced after resistance wheel exercise, and it is associated with increased muscle size in adulthood (Smith and Merry, 2012).

When submitted to tension, the amount and duration of the stimulus can cause lesions in the myofibres (Lieber et al., 2000; Caiozzo, 2002). Such lesions, triggered by immobilisation or physical or therapeutic exercises, alter cellular size and form and can impair muscle function. Canu et al. (2005) reported changes in gait after 14 days of microgravity simulation in adult rats, including a longer support phase, increased step length, reduced hindlimb extension at the moment of initial contact, and a tendency towards hip abduction. In addition, we demonstrated that intermittent stretching for six days after immobilisation in adult rat helped re-establish step length to pre-immobilisation values (Cação-Benedini et al., 2013).

Therefore, due to the high prevalence of disorders affecting neuromuscular development and the lack of morphofunctional reports supporting specific therapies, there is a need to compare different rehabilitation methods. Thus, using active and passive stimuli in weanling female rats, this study analysed functional alterations and morphological elements involved in force transduction and regeneration following immobilisation and rehabilitation.

Materials and methods

Animals

This study was approved by the Commission of Ethics in Animal Experimentation of the Ribeirão Preto Medical School, University of São Paulo. Fifty-six Wistar female rats, 21 days old, were used; this corresponds to the weaning phase. The animals were supplied by the university's Central Animal Facilities and were divided into cages with four animals each. The animals had free access to water and food, and the cages were sanitised twice daily, in agreement with animal facility procedures. The experimental protocols and animal care and treatment were approved by the Animal Ethics Committee of Ribeirão Preto Medical School (Process 175/2010).

Experimental procedure

The animals were divided randomly into one of the following groups: I (immobilised with no rehabilitation, n=4), IiS (immobilised and rehabilitated by intermittent stretching, n=8), ImS (immobilised and rehabilitated by maintained stretching, n=8), IE (immobilised and trained eccentrically, n=8), IF (immobilised and maintained free in the cage, n=8), A (anesthetised, n=4), C21 days old (control group aged 21 days, n=4), and C31 and C34 days old (control groups aged 31 days, for comparison with the I group, and aged 34 days, for comparison with rehabilitated groups, respectively; n=4 each).

Immobilisation technique

The immobilisation procedures were performed according to Benedini-Elias et al. (2009), with the tibio-tarsal articulation of the weanling rats maintained at maximum plantar flexion for 10 consecutive days. The animals were previously anesthetised intraperitoneally with chloral hydrate (4%). Immobilisation did not prevent feeding or locomotion inside the cage, which was sanitised daily. The immobilisation device was removed on the tenth day, the same day in which the proposed rehabilitation techniques were initiated for three consecutive days.

Rehabilitation protocols

Intermittent Stretching Technique: IiS animals underwent intermittent manual passive stretching. The stretching was accomplished by applying manual force (not quantified) to the plantar portion of the right hindlimb to generate dorsiflexion with the knee in full extension; this generated passive stretching of the soleus and plantaris muscles (Mattiello-Sverzut et al., 2006). The stretching exercises were performed in a single series of ten exercises of 30 seconds each, with a 30-s interval between exercises.

Maintained Stretching Technique: ImS animals underwent daily maintained stretching. The technique consisted of fixing the ankle in dorsiflexion with adhesive tape, as described by Gomes et al (2007), for 20 minutes on the first day, 30 on the second, and 40 on the third. All animals were previously anesthetised intraperitoneally with chloral hydrate at 4%.

Eccentric Exercise Training: IE animals were submitted to daily training by downhill treadmill running (Insight® model EP-131) with a 16° slope and a speed of 17 m/min for 20 minutes on the first day, 30 on the second, and 40 on the third (Cornachione et al., 2008).

Functional analysis of gait

Prior to immobilisation, the animals underwent functional analysis of gait; the analysis was repeated on each day during the rehabilitation or free movement protocol. The system for collecting videos used for analysis included treadmill enclosed in acrylic and metallic structure (Insight® of Ribeirão Preto). The portion of the treadmill where the animals ambulated was prepared using transparent polycarbonate canvas; this allows the filming of the animal's gait using a 1.3-megapixel webcam coupled to a laptop computer. Before immobilisation, the animals were acclimated to the treadmill for approximately five minutes each on two days. After the acclimation period, three-minute videos were made to record the control data for gait. After ten days of immobilisation, the animals were filmed again for three consecutive days during the respective rehabilitation or free movement protocols. This analysis was always accomplished after the rehabilitation procedures.

From the footage, images of the footprints were selected in the double-support phase, with the right hindlimb responding to the load and the left hindlimb on pre-swing (Varejão et al., 2002). Images were re-sized to a suitable size with Adobe Photoshop software, version CS3®. The following points were then labelled: length of the right footprint (PLR), length of the left footprint (PLL), total opening of the toes of the right hindlimb, measured as the distance from the first to fifth toes (TS) and the opening of the intermediate toes of the right hind limb, from the second to fourth toes (IT), as suggested by Bain et al. (1989). At the marking of the last pressure points of PLR and PLL, two perpendicular straight lines were drawn to determine step length (SL) and support base (SB). These values were obtained manually using ImageJ® software, which converts the measured values from pixels to centimetres.

Histochemistry

At the end of the experimental period, the animals were weighed and euthanised with excess sodium thiopental (Thiopentax). The soleus and plantar muscles were dissected, removed, weighed, covered with talc, and later frozen in liquid nitrogen. Transverse sections (5-μm thickness) were obtained using a cryotome (Leica Microsystems, Frankfurt, Germany) at −25°C, mounted on 26x76 mm slides, and stained with haematoxylin-eosin (HE).

Immunohistochemistry

Fibronectin immunoreactivity was obtained using primary goat polyclonal anti-human fibronectin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Types I and III collagen were identified using primary mouse anti-rat type I and type III collagen antibodies (Sigma-Aldrich, St. Louis, MO, USA). The slides were then fixed in ice-cold acetone for 10 minutes, washed in phosphate-buffered saline (PBS), and incubated in 1% H_2O_2 solution for 15 minutes to block endogenous peroxidase activity. The slides were washed again in PBS and then in Tris-buffered saline with 0.05% Tween 20 (TBST) for five minutes. Subsequently, non-specific binding was blocked by incubation with 2% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) and normal goat serum (Vector S-1000, Vector Laboratories, Burlingame, CA, USA) for 20 minutes. The excess liquid was removed, and the samples were incubated with primary mouse anti-rat type I collagen (1:18.000 dilution), mouse anti-rat type III collagen (1:36.000), or goat polyclonal anti-human fibronectin (1:100) antibodies at 4°C overnight. The following day, the slides were washed in TBST for nine minutes and incubated with the secondary antibody (1:200) from the Vector kit (PK6200, Vector Laboratories, Burlingame, CA, USA) for 30 minutes. The slides were washed in TBST and then incubated for 30 minutes in avidin + biotin from the Vector kit (PK6200). The slides were washed again in TBST and incubated with diaminobenzidine (DAB) chromogen (Sigma-Aldrich, St. Louis, MILLSTONE, USA) for five minutes. Finally, the slides were washed with distilled water, counterstained with haematoxylin (Merck, Darmstadt, Germany) for 10 seconds, dehydrated, diaphanised, and mounted in Permount (Fisher Scientific, Fair Lawn, NJ, USA).

Double labelling was carried out using laminin + Pax-7 and dystrophin + CD68. The slides were briefly washed in PBS and fastened in Xpress molecular fixative (Sakura Finetek, Alphen aan den Rijn, Holland) for four minutes, then washed three times in PBS for

five minutes each. The slides were then blocked with 10% goat serum (Vector Laboratories, Burlingame Road, California, USA) in PBS for 60 minutes; slides were then blocked with Avidin for 15 minutes (Avidin/Biotin Blocking Kit, Vector Laboratories, Burlingame Road, California, USA), with a subsequent brief wash in PBS. After removing the excess liquid, the samples were incubated with primary rabbit polyclonal anti-laminin (1:100; Abcam, Cambridge, UK) + mouse Pax-7 (1:500; Santa Cruz) antibodies or a mix of rabbit polyclonal anti-dystrophin (1:400; Abcam, Cambridge, UK) + mouse monoclonal [ED1] anti-CD68 (1:200; Abcam, Cambridge, UK) antibodies at 37°C for two hours. The slides were subsequently washed three times in PBS for five minutes each, incubated with fluorescent goat anti-rabbit green 488 + goat anti-mouse red 56 (1:1,000; Invitrogen, New York, USA) secondary antibodies for 45 minutes. Both the slide and the dystrophin were marked in green, and Pax-7 and CD68 were marked in red. Three additional washes with PBS of five minutes each were carried out, and the slides were applied with Prolong Gold Antifade (Invitrogen, New York, USA).

Morphometry

Qualitative analyses of the histological slides were performed using a Microscope of Light (ML) Leica DM 2500 coupled with a DFC 300FX camera. General muscle morphology was evaluated by HE staining and fibronectin immunohistochemistry. The morphometric analysis used the interactive mode of LAS software using three images from each animal; the images were captured with the optical microscope and camera connected to a microcomputer as previously mentioned. The total number of Pax-7+ cells was obtained by singly counting intracellular and extracellular Pax-7-stained cells. Laminin labelling defined the limits of satellite cell position. This method was also used to count CD68+ macrophages where dystrophin labelling defined the cell membrane. Semi-quantitative analysis, as proposed by Kurose et al. (2006), was used to evaluate types I and III collagen expression levels. Three independent examiners using the optical microscope described above analysed the tissue images based on the following reactivity classification scheme: absent (–), weakly positive (±), slightly positive (+), moderately positive (++), or strongly positive (+++). The evaluators were blinded to the identity of the treatment groups.

Statistical analysis

To statistically analyse data obtained with Pax-7+ and CD68+ cells, the Mann-Whitney test was used with a significance level of 5%, using the GraphPad Prism 5 statistical program (GraphPad Software Inc., San Diego, CA, USA). Gait-related variables were analysed using the linear mixed-effect model with the significance level (α) set at 5% and the confidence interval (CI) at 95%; the model adjustment was performed using SAS 9.2 software (SAS, Wiley, New York, USA).

Results

Weight increased less in immobilised animals (I, ImS, IiS, and IE) than in C31, with final weights of 71.05±2.13 g and 159.37±10.27 g, respectively. After rehabilitation therapies or free movement in the cage, the animals gained considerable weight ($p<0.05$), although it was less than C34 (final weights: IE, 113.65±9.11 g; ImS, 102.88±8.3 g; IiS, 95.0±14.96 g; IF, 118.68±14.07;

Table 1. Gait functional parameters: Description of mean values.

Groups	Period	Support Base	Step Lenght	Intermediate Toes	Print Lenght Right	Print Lenght Left	Spread of Toes
IF	Pre-i	1.60	4.00	0.80	1.00	0.90	1.30
	1st post-i	0	0	0.01	0	0.84	0.02
	2nd post-i	0.04	0.08	0.07	0.02	0.87	0.10
	3rd post-i	0.14	0.62	0.07	0.09	0.87	0.09
IE	Pre-i	1.61	3.47	0.77	1.13	0.92	1.32
	1st post-i	0	0	0	0	0.88	0
	2nd post-i	0.64	1.54	0.32	0.43	0.86	0.42
	3rd post-i	0.80	2.40	0.44	0.56	0.86	0.69
IiS	Pre-i	1.63	3.50	0.68	0.88	0.88	1.20
	1st post-i	0.54	1.29	0.16	0.31	0.27	0.37
	2nd post-i	0.98	1.98	0.36	0.52	0.37	0.56
	3rd post-i	1.11	2.65	0.56	0.75	0.55	0.86
ImS	Pre-i	1.59	3.72	0.70	0.98	0.90	1.28
	1st post-i	0	0	0	0	0	0
	2nd post-i	0	0	0	0	0	0
	3rd post-i	0	0	0	0	0	0

Data are expressed as means with their respective 95% confidence intervals below in parentheses. cm, centimeters; IF, immobilized and free; IE, immobilized and trained eccentrically; IiS, immobilized and intermittent stretched; ImS, immobilized and maintained stretched; pre-i, pre-immobilization period; post-i, days post-immobilization; *, $p<0.05$.

and C34, 172.63±12.09 g).

Gait-related variables were not statistically different among the groups prior to immobilisation (Table 1). Immobilisation of the right hindlimb prevented dorsiflexion and kept the hip and knee flexed, thus impeding the weight-bearing and support phases in the right hindlimb in immobilised animals. During immobilisation, gait occurred predominantly with forelimb movement that impelled the animal forward.

On the first day after the respective rehabilitation protocols, few animals were able to bear weight on the right hindlimb. The video images showed that the initial contact during gait occurred by supporting or dragging the lower limb or by overlapping the tips of the toes at some point. During the weight-bearing phase of gait, the rear left hindlimb presented internal rotation and greatly increased space among the toes, increasing the contact area with the ground surface. On the second day, the IE group presented a support phase; however, the right hindlimb presented difficulty in crossing the left hindlimb in the swing phase, which reduced the SB and increased the SL in relation to the initial values. PLR and IT remained lower than initial values. On the third day, most of the IE, IF, and IiS animals were able to touch the ground surface with the toes of the right hindlimb; however, SB continued to be significantly reduced. The SL medium values continued to increase, but did not reach the initial values, and ST also remained significantly lower. The ImS animals did not touch the ground surface with their right hindlimb.

A significant reduction in muscle weight occurred in all immobilised groups. Morphological analysis of the soleus muscle showed minimal signs of inflammation and isolated necrosis, reduced muscle fibre volume, and irregularity in shape compared with the control group (Fig. 1a). When comparing experimental groups, a higher degree of involvement of the soleus muscle of the ImS group can be observed, followed by the IE and IiS groups (Fig. 1b,c, respectively). The soleus of the IE group presented rounded fibres, increased interstitial space and cellularity, centralised nuclei, delta lesions, and inclusion bodies (Fig. 1b). IiS presented with similar abnormalities, but to a lesser extent (Fig. 1c), while ImS showed them to a greater extent (Fig. 1d). Muscles of the IF group presented fewer abnormalities than the IiS group, and the anesthetised animals (A) showed no muscle fibre abnormalities (not shown). Plantaris muscle fibres generally showed lower reactivity than the soleus and were also rounded and/or polyhedral, with centralised nuclei, but without significant tissue

Table 2. Gait functional parameters: Comparisons intra and intergroups between the pre- and post-immobilization periods.

Groups	Support Base	Step Lenght	Intermediate Toes	Print Lenght Right	Print Lenght Left	Spread of Toes
	Pre-i x 3rdpost-i	Pre-i x 3rdpost-i	Pre-i x 3rdpost-i	Pre-i x 3rdpost-i	Pre-i x 3rdpost-i	Pre-i x 3rdpost-i
IF	1.60 x 0.14*	3.95 x 0.62*	0.75 x 0.07*	1.03 x 0.09*	0.99 x 0.87*	1.26 x 0.09*
IE	1.60 x 0.80*	3.47 x 2.41	0.77 x 0.44*	1.13 x 0.56*	0.92 x 0.86	1.32 x 0.69*
IiS	1.59 x 1.11*	3.57 x 2.65	0.68 x 0.56	0.88 x 0.75	0.87 x 0.55	1.20 x 0.86
ImS	1.60 x 0*	3.70 x 0*	0.70 x 0*	0.90 x 0*	0.90 x 0*	1.22 x 0*
	3rdpost-i	3rdpost-i	3rdpost-i	3rdpost-i	3rdpost-i	3rdpost-i
IiS x IF	1.12 x 0.14	2.65 x 0.62*	0.56 x 0.07*	0.75 x 0.09*	0.55 x 0.87	0.86 x 0.09*
IiS x IE	1.11 x 0.80	2.65 x 2.41	0.56 x 0.44	0.75 x 0.56	0.55 x 0.85	0.86 x 0.69
IF x IE	0.14 x 0.80	0.62 x 2.41*	0.07 x 0.44*	0.09 x 0.56*	0.87 x 0.86	0.09 x 0.69*

Data are expressed as means with their respective 95% confidence intervals below in parentheses. cm, centimeters; IF, immobilized and free; IE, immobilized and trained eccentrically; IiS, immobilized and intermittent stretched; ImS, immobilized and maintained stretched; pre-i, pre-immobilization period; post-i, days post-immobilization; *, p<0.05.

Table 3. Semi-quantitative evaluation of collagen types I and III.

Soleus	21	31	34	I	IF	ImS	IiS	IE	A
Collagen I	+++	+	+	++	++	++	+/++	++	±/+
Collagen III	++	+	+/++	++/+++	++/+++	++/+++	++/+++	++	+/++
Plantaris	21	31	34	I	IF	ImS	IiS	IE	A
Collagen I	++/+++	++	+	+	++	+/++	++	+/++	+
Collagen III	++/+++	++	+	++	++	+	++	+/++	++

±, weakly positive; +, slightly positive; ++, moderately positive; +++, strongly positive. C21, control 21 days-old; C31, control 31days-old; C34, control 34 days-old; IF, immobilized and free; IE, immobilized and trained eccentrically; IiS, immobilized and intermittent stretched; ImS, immobilized and maintained stretched; pre-i, pre-immobilization period; post-i, days post-immobilization.

abnormalities (not shown).

Immunohistochemical analysis of fibronectin indicated the presence of fibronectin only in the interstitial spaces of the soleus and plantaris of all groups.

In soleus, immobilisation triggered an isolated increase in types I and III collagens expression; however, the ratio between them did not change. Even the therapeutic exercises or free movement in the cage for three days did not change the ratio of collagens I and III (Table 3). There was an increase in Pax-7+ nuclei in the intra- and extracellular regions of the IF, IE, and ImS groups compared to I ($p<0.05$, Table 4). Plantaris fibres generally showed less Pax-7+ nuclei in the intra-and extracellular regions, but only ImS showed a significant increase in Pax-7+ nuclei compared to C34 ($p<0.05$).

In soleus and plantaris muscles, the absence of intracellular macrophages was observed in the analysed groups. A significant increase in CD68+ cells occurred extracellularly in the soleus muscle in all groups undergoing rehabilitation and unrestricted movement when compared with C34 ($p<0.05$, Table 5). For the plantaris, a significant increase was observed only in IE compared to C34 ($p<0.05$, Table 5).

Discussion

This study followed weanling female rats that had the right hindlimb immobilised for 10 days and were subsequently submitted to different rehabilitation protocols for three consecutive days; the aim was to analyse possible functional and morphological differences among the recovery procedures.

The immobilised groups had a lower weight than the

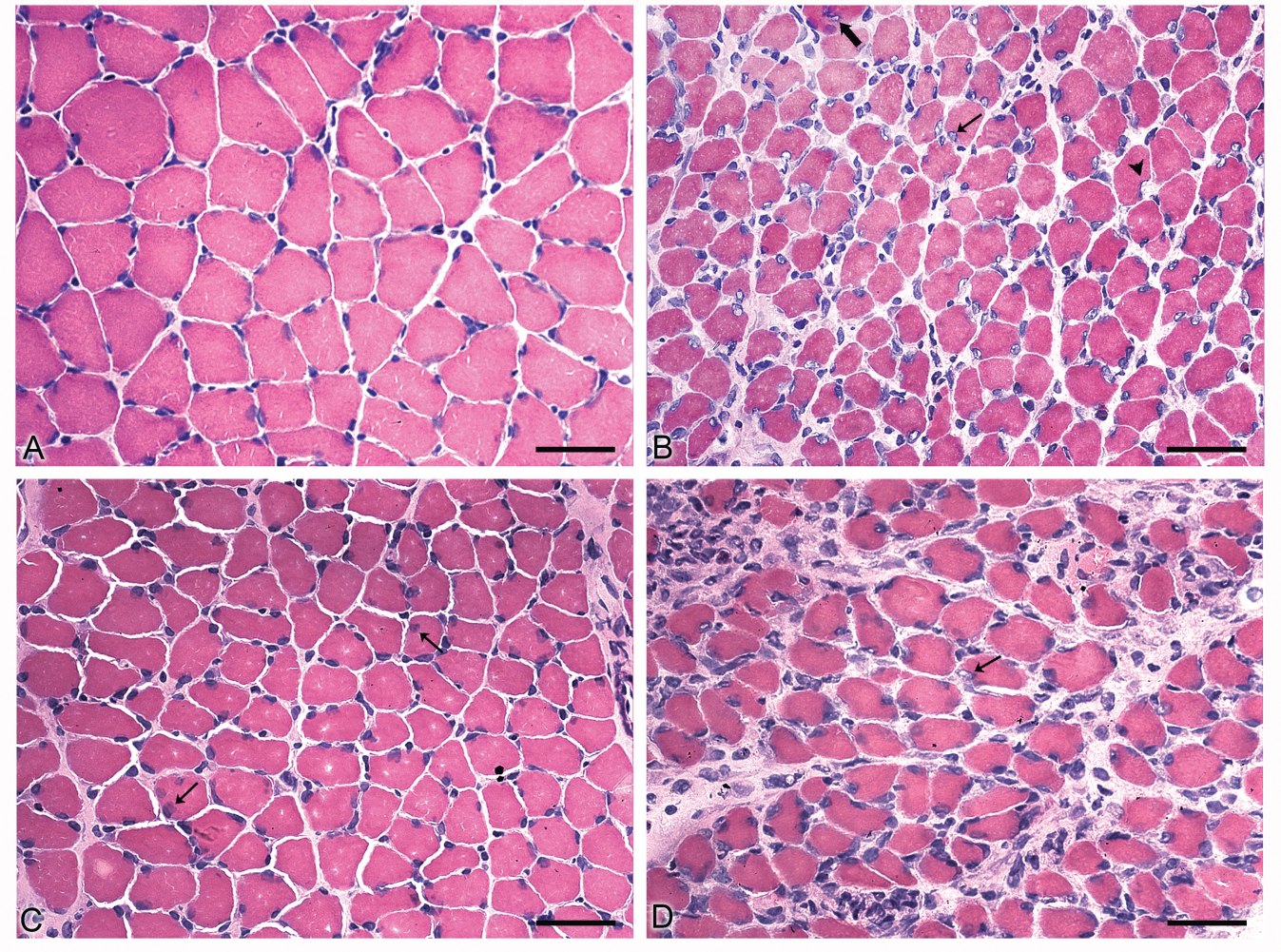

Fig. 1. Photomicrographs of the soleus muscle, stained with haematoxylin-eosin **(A–D). A.** (C34): polyhedral fibres and peripheral nuclei. **B.** (IE): rounded fibres, increased interstitial cellularity, inclusion bodies (thin arrow), nuclear centralisation (thick arrow), and cells with delta lesions (arrowhead). **C.** (IiS): fibres with smaller size and inclusion bodies (thin arrow). **D.** (ImS): increased interstitial cellularity, necrotic fibres, and inclusion bodies (thin arrow). Bars: 40 µm.

Table 4. Medium values and standard deviation of Pax-7+ nuclei in the muscles analyzed.

Soleus	C21	C31	C34	I	IF	IE	ImS	IiS	A
Intracelular	0 (0)*	1.50 (1.09)*	0.58 (0.79)	1.71 (3.65)†	0.92 (1.17)°	1.77 (1.30)*°†	0.88 (1.50)	2.55 (2.43)*°†	1.58 (1.62)
Extracelular	1.92 (1.93)*	1.00 (1.13)	0.08 (0.29)	0.52 (0.98)	2.33 (2.18)*	3.47 (3.06)*°◊	2.33 (2.23)*°	3.30 (2.49)*°	0.83 (0.83)*◊
Plantaris	C21	C31	C34	I	IF	IE	ISi	ISs	A
Intracelular	0.75 (1.29)	1.08 (0.99)*°	0.17 (0.58)	0,09 (0,44)†	0.54 (0.93)°	0,27 (0,58)◊	0,07 (0,27)†◊	0,85 (0,99)*°	0.08 (0.29)◊
Extracelular	0.08 (0.29)	0.08 (0.29)	0.08 (0.29)	0.90 (1.41)	0.92 (1.10)*	1.76 (1.50)*°†	0.41 (0.75)†◊•	1.22 (1.50)*	1.16 (1.34)*

Mann-Whitney test ($p<0,05$): * compared with C34; ° compared with I; † compared with IF, ◊ compared with ISs, • compared with IE. C21, control 21 days-old; C31, control 31days-old; C34, control 34 days-old; IF, immobilized and free; IE, immobilized and trained eccentrically; IiS, immobilized and intermittent stretched; ImS, immobilized and maintained stretched; pre-i, pre-immobilization period; post-i, days post-immobilization.

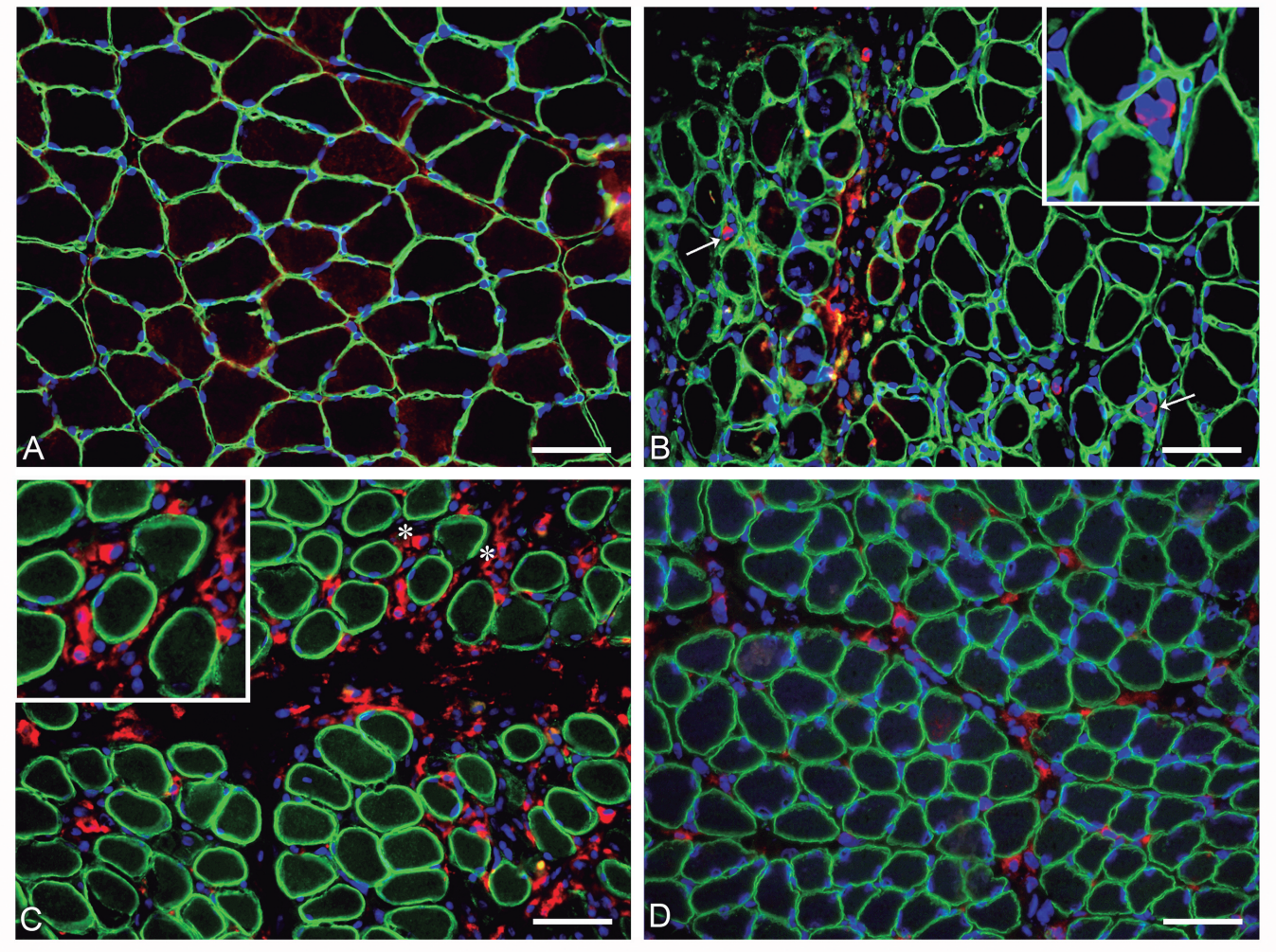

Fig. 2. Photomicrographs of the soleus muscle with a double-staining pattern, merged images. **A, B.** anti-Pax-7 and anti-laminin antibodies. **C, D.** anti-CD68 [ED1] and anti-dystrophin. **A.** (C34): observe polyhedral fibres and peripheral nuclei. **B.** (ImS): observe Pax-7+ cells (arrows, amplified) and increased interstitial cellularity. **C.** (ImS): observe intense labelling of CD68 and increased interstitial cellularity (asterisk, amplified). **D.** (IE): observe positive labelling of CD68 and interstitial cellularity. Laminin: green; Pax-7: red; dystrophin: green; CD68: red; adult nuclei: blue. Bars: 40 µm.

Table 5. Medium values and standard deviation of CD68+ cells in the muscles analyzed.

Soleus	C21	C31	C34	I	IF	IE	ImS	IiS	A
Intracelular	0.10 (0.29)	0	0.10 (0.29)	0	0.30 (0.70)°	0	0†	0 (0.90)†	0 (0.30)◊
Extracelular	2.42 (1.73)	1.83 (0.94)	1.75 (2.05)	4.17 (2.63)	9.43 (6.82)*	8.10 (5.88)*	6.74 (5.91)*†°	6,70 (5.86)*	2.42 (2.31)
Plantaris	C21	C31	C34	I	IF	IE	ISi	ISs	A
Intracelular	0	0	0	0.04 (0.20)	0	0	0	0	0
Extracelular	0.42 (0.99)*	1.50 (1.78)	2.08 (1.73)	1.92 (1.53)	2.76 (1.84)°	4.60 (2.39)*†	2.63 (2.34)°•	5.19 (3.80)°	0.42 (0.99)*◊

Mann-Whitney test (p<0,05): *compared with C34; ° compared with I; † compared with IF, ◊ compared with ISs, • compared with IE. C21, control 21 days-old; C31, control 31days-old; C34, control 34 days-old; IF, immobilized and free; IE, immobilized and trained eccentrically; IiS, immobilizes and stretched intermittent; ImS, immobilized and maintained stretched; pre-i, pre-immobilization period; post-i, days post-immobilization.

control group, suggesting that the immobilisation device negatively influenced growth. This adapted immobilisation device (Benedini-Elias et al., 2009) used low-weight materials and is easy to sanitise, easy to handle, adjustable for the growing animals, and allows the animal to move around and feed during immobilisation. Furthermore, this model induces atrophy and restriction of joint movement as well as other devices presented in the literature, such as casts or plastic gutters; however, with lesser impediment to development (Coutinho et al., 2004; Gomes et al., 2007). After removing the device, weight increased in IF, IE, and ImS. ImS were anesthetised and presented larger weight gains than IiS. It is possible that the stress generated by extra handling of the IiS group might have interfered in feeding, as IiS did not gain as much weight as ImS.

Immobilisation of the right hindlimb altered gait in an important way. After removing the immobilisation device, the animals adopted the same posture as when they were immobilised: hip and knee extended and plantar flexion, not supporting the right hindlimb. This can be explained by the joint restriction and posture adopted during the 10 days of immobilisation. Regarding rehabilitation, IiS and IE seemed to come closer to the pre-immobilisation values than those in IF. There was a delay in ImS in supporting weight on the right hindlimb during gait. These functional findings agreed with the morphologic ones, where ImS and IE presented larger and smaller degree of alterations in the muscle tissue, respectively. As maintained stretching presents more lesions than the eccentric training, this can justify why maintained stretching postpones bearing weight on the right hindlimb during gait.

On the third day of rehabilitation, the animals continued to increase SL, probably to compensate for the reduced BS, trying to achieve stability. TS remained reduced in all groups, and ImS values approached closer to the initial values. This demonstrates that intermittent stretching as rehabilitation provided more proximity to the normal functional conditions following hindlimb immobilisation.

The final IF data remained different from the initial values and from ImS ($p<0.05$), demonstrating the benefits of three days of eccentric exercise and intermittent stretching for re-establishment of functional performance after immobilisation. The literature shows the advantages of active rehabilitation in adult and weanling rats over a long period of rehabilitation (Kannus et al., 1998; Cornachione et al., 2014; Cação-Benedini et al., 2013). The present data is innovative in that it compares functional and morphologic data in weanling rats and further compares four different rehabilitation procedures over a short period.

Several studies report disuse-associated reductions in muscular weight (Mozdziak et al., 2000; Okita et al., 2004; Frimel et al., 2005). The present study showed that immobilisation performed as described herein induced a 35% reduction in soleus weight and a 28% reduction in plantaris weight. Disuse results in an imbalance between the synthesis and the degradation of myofibrillar proteins, reducing protein content and muscle volume. In addition, immobilisation reduces signal transduction in the muscle fibres, which reduces protein synthesis and increases proteolysis (Powers et al., 2005). Nutrition also favours muscle protein synthesis in neonates, which promotes increased muscular protein mass and, consequently, an increase in muscle weight (Davis and Fiorotto, 2009). After removal of immobilisation, facilitated access to feeding may have contributed towards the increased muscle weight.

The stretched groups (passive tension) presented more necrotic and inflammatory signs when compared with the actively trained group (eccentric exercise). It is known that maintained stretching and eccentric exercise can cause microinjuries in the muscle fibres (Yang et al., 1997). The hypotheses generated to explain these data are as follows: (1) the force employed for stretching

protocols was manual and was not measured; this might have induced more cellular lesions than those from the treadmill protocol; (2) the treadmill protocol is active, and the animals could modulate their effort, thus perhaps minimizing the muscle injury and preserving their fibre cytoarchitecture.

Immobilisation increased collagen I and II expression in the soleus, in agreement with findings from adult animals (Jozsa et al., 1988). The observed alterations are generated by changes in the synthesis of muscular proteins and collagens (Karpakka et al., 1990); this is due to the fast and dynamic adaptation of muscle collagens to the presence or absence of mechanical load (Mackey et al., 2008). Type I collagen is composed of parallel fibres, which furnish tensile force and rigidity to the tissue, and type III collagen provides compliance (Salonen et al., 1985; Kurose et al., 2006). Changes occurring in the intramuscular connective tissue after immobilisation, such as the altered relationship of expression between types I and III collagen, may contribute to the functional loss and alterations in the biomechanical properties of skeletal muscle, since the properties of tensile force, rigidity, and muscular compliance are modified. However, the present study revealed no significant alteration in the ratio between the expressions of collagens I and III after immobilisation or during the rehabilitation protocols. Thus, it seems that the gait abnormalities and cellular injury observed in the muscles of these animals cannot be due to passive extracellular components. Other factors must be involved in the gait quality observed in immobilised and rehabilitated animals. Such factors are situated in the muscles, nerves, neuromuscular junction, and medulla. For example, Salanova et al. (2011) demonstrated that chronic inactivity changes the expression of the neuromuscular protein Homer2, which is neurally modulated.

Intracellular positive labelling of fibronectin in muscle fibres from adult animals was demonstrated by our research group (Cação-Benedini et al., 2013). Other scientific studies have used weanling animals, but fibronectin in skeletal muscle was not reported. It is hypothesised that developing muscle tissue could respond differently than adult tissue. For example, Goldspink et al. (1992) reported that muscles in development presented more extensive adaptations to reduced muscular activity than adults, and may need more time or greater intensity of the rehabilitation protocols to revert these adaptations.

The significant increase of extracellular macrophages in the soleus demonstrates the degenerative process evoked by immobilisation with rehabilitation. The intensity of the inflammatory responses seems to have not been influenced by the current rehabilitation protocols as chemical events activating macrophages seemed to occur (Tidball, 2005). The amount of extracellular macrophages increased in all rehabilitated groups. In the soleus muscle, IF seemed to allow greater inflammation when compared with IiS, potentially indicating that the rehabilitation protocols can modulate this process. A previous study with adult rats also showed an increased number of macrophages in the soleus muscle of free and stretched rats three days after immobilisation (Cação-Benedini et al., 2013).

Satellite cells of the immobilised muscles did not suffer significant alterations when compared with the control group. Studies show that the satellite cell mitotic activity in young muscles is suppressed after 24 hours from the beginning of caudal suspension, and it is maintained throughout suspension (Darr and Schultz, 1989; Schultz et al., 1994). The hypomobility-generated muscular atrophy seems not to interfere in the cycle of the satellite cells, justifying the present data. Snijders et al. (2014) observed the satellite cell content of type I or type II fibres was not modified after immobilisation During the regeneration process, satellite cells in very young animals can be mobilised more easily (Davis and Fiorotto, 2009). The centralised nuclei, verified in our morphologic findings, probably are derived from satellite cells, and the increase in centralised nuclei in both the free and rehabilitated groups demonstrates the muscle tissue regeneration promoted by remobilisation (Oustanina et al., 2004). Järvinen et al. (2005) reported that on the third day after lesion, there was activation of the satellite cells, which release several substances responsible for amplifying the inflammatory reaction; this was observed in our study by the increase of macrophages present in the interstitial fluid. Hwang et al. (2006) verified that during 16 days of passive stretching, it is possible to maintain the activation and proliferation of satellite cells after disuse by caudal suspension, which reduced activated and quiescent satellite cells and the number of myonuclei. Another study verified a significant increase of satellite cells in human type I and II muscle fibres after only a single session of eccentric exercise (Snijders et al., 2012). The IE, with a larger amount of satellite cells in both muscles, also had a better morphologic aspect, activating great amounts of extracellular macrophages, and also presented good functional performance during the gait analysis.

The plantaris was consistently less reactive to disuse and remobilisation stimuli, not presenting important adaptive alterations. In a previous study with female weanling rats we verified that the glycolytic characteristic of the plantaris presents less intense necrotic and inflammatory post-immobilisation responses with training than oxidative muscles (Benedini-Elias et al., 2014).

We conclude that among the four rehabilitation procedures used with recently weaned female rats, active exercise, represented here by eccentric training, resulted in better morphologic and functional results. Training by intermittent stretching was the second-most effective, and the least effective training methods were maintained stretching and free movement in the cage. We emphasize the need for more studies in this development phase, which seems to respond particularly well to external

stimuli such as disuse and remobilisation.

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