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Original Article

Acute Electrical Stimulation Modifies Cross-sectional Area and Desmin Protein in the Skeletal Muscle of Old Rats Submitted to Hindlimb Suspension

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Abstract

The aim was to determine the acute effects of high-frequency electrical stimulation (HFES) on the morphohistology and desmin content of the soleus, tibialis anterior (TA) and *extensor digitorum longus* (EDL) muscles in old rats submitted to hindlimb suspension (HS). Male Sprague-Dawley rats (23 months-old) were submitted to HS for 14 days. After this period, the Control and HS groups underwent a single session of HFES. HS decreased muscle mass and CSA-MF in all the muscles and desmin content in the soleus and EDL muscles, but increases its content in the TA muscle in the control group. HFES increased the CSA-MF in the soleus muscle and decreased it in the TA and EDL muscles in the control group. HFES also increases the desmin content in the soleus and EDL muscles, but decreases it in the TA muscle. One session of HFES has different effects on the morphohistology of the soleus, TA and EDL muscles in this study.

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Introduction

Skeletal muscle is a plastic tissue (1) which may be affected by inactivity, inducing muscle disuse atrophy with morphohistology changes resulting in a loss of muscle mass and decrease in the cross-sectional area of muscle fibers (CSA-MF) (2, 4-7). Several studies noted that after periods of disuse or denervation there is a decrease in the proportion of type I fibers and increased of type II fibers (1, 5, 8). The intracellular mechanisms of muscle atrophy that are associated to aging were not extensively studied, but they appear to be different from those induced by cachexia or inactivity (9). Aging is associated with a progressive decline of mass, strength and quality of the skeletal muscle, a condition described as sarcopenia (10, 11). During aging, the reduction of type II CSA-MF is around 20 to 50%, while loss of type I CSA-MF is between 1 to 25%, supporting that aging is associated with a specific muscle phenotype change, which may be associated to a reduction of neuromuscular stimulation (10, 11). Because of the complexity involved in investigating the mechanisms responsible for muscle atrophy in humans, several experimental models in animals were developed (12). One of the most used is the hindlimb suspension (HS)-animal model performed in animals of different ages (7, 12-15). This experimental model mimics muscle disuse atrophy that occurs in conditions of bed rest, hospitalization or space flight in humans. The study by Hwee and Bodine (2009) demonstrated that, when performing HS for 14 days in young (6 months) and old rats (30 months), the percentage of loss due to HS for the soleus muscle mass was of 42% and 34%, for the TA muscle 19% and 14% and for the EDL muscle 13% and 14%, respectively (16). Leeuwenburgh et al. (2005) observed the difference of soleus muscle mass between groups of young (6 months) and old (32 months) rats. They detected a decrease of 24% and 26%, respectively, after 14 days of HS. The CSA-MF of soleus muscle decrease 58% after HS in old rats when compared with the respective control old group in the same study (8). Chronic exposure of skeletal muscle to mechanical stress tends to be presented as the most desirable way to mitigate the effects of sarcopenia (17, 18). Strategies such as oral nutritional supplementation, physical activity or muscle contraction produced by high-frequency electrical stimulation (HFES) could be the most effective ways to counteract the effects that are often observed in the skeletal muscle disuse atrophy (17, 18). The model of *in vivo* HFES (100 Hz) elicits muscle protein synthesis and hypertrophy. Thus, different authors try to reverse muscle atrophy in rats (18-21) and humans (22, 23) using HFES.

The major component of the intermediate filaments of adult muscle is the desmin protein (470 amino acids) (24). It weights 53 kDa, exhibits a diameter of 8-14 nm (25) and is expressed in skeletal, cardiac and smooth muscles, being more abundant in cardiac muscle (2% of total protein) that in the skeletal muscle (0.35% of total protein) (24). Its interaction with other proteins forms a continuous network in the cytoskeleton that provides a spatial relationship between the contractile apparatus and other structural elements of the cell, allowing the maintenance of integrity, efficient force transmission and mechanochemical signaling within the fiber (24, 26). The alteration of this protein affects the regular functioning of the muscle cell. Ansved and Edström (1991), performing immunohistochemistry in the soleus muscle of young and old rats, observed that the desmin content increased in old rats because the loss of myofibrils leaves more space for the accumulation of desmin (27). Also, Russ and Grandy (2011) observed an increase in the desmin protein measured by immunoblotting in old rats (28). On the other hand, Meyer and Lieber (2012) observed a decrease in the expression of desmin protein in healthy adult subjects compared to young ones (29). Regarding the levels of desmin protein during HS periods, there are discrepancies. Chopard et al. (2001), in 6 weeks-old rats, performing measurements by western blotting, observed no change in the desmin content at 3 and 6 weeks of HS in the soleus and *extensor digitorum longus* (EDL) muscles (30). Furthermore, Ogneva (2010), also with the use of western blotting, observed that the effects of HS on desmin content in young rats is dependent of muscle type and disuse duration. On the first days (i.e., 3-7 days) of HS, desmin content decreases in the soleus muscle. However, as HS is maintained for longer periods (i.e., 14 days), the content of this protein increases. The opposite occurs in the medial

gastrocnemius and tibialis anterior (TA) muscles (31).

An individual who suffers muscle atrophy by aging usually has decreased functionality, further increasing his muscle wasting due to disuse. In this study we combine these situations: old rats submitted to HS (aging + disuse). Taking into account the discrepancies mentioned above, the objective of this study was to determine the acute effects of HFES on CSA-MF and desmin content of soleus, TA and EDL in old rats submitted to HS.

Methods

Animals

Twenty three months-old male Sprague Dawley rats were housed individually under standardized climate controlled conditions: 12-hour light/dark cycles with room temperature of 22–24°C and food and water consumption *ad libitum* (recorded daily). All experimental procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Washington, DC, USA). The study was approved by the Ethics Committee of the Catholic University of Maule, Talca, Chile (n° 03.2011).

Study Design

During the first three days of the experimental period, all animals were adapted to individual cages. Afterwards, the animals were divided randomly into two groups: control group (C, n=6) and HS group (HS, n=6). HS group began with the suspension of their hind limbs on day 1. HS continued for 14 days, and in the 15th day the animals of both groups were weighed and anesthetized with intraperitoneal injection of ketamine (75 mg/kg) and xylazine (5 mg/kg). Then, each animal underwent a single session of HFES of 30 minutes in the right limb defining four groups (control group [C]; electrical stimulation control group [ES-C]; hindlimb suspension group [HS] and electrical stimulation + hindlimb suspension group [ES-HS]). Following this, the soleus, TA and EDL muscles from both hind limbs were isolated, weighed and stored. Finally, rats were sacrificed with lethal dose of intracardiac sodium pentobarbital.

Animal model of hindlimb suspension (HS)

The HS group underwent a noninvasive technique that produces skeletal muscle atrophy in the muscles of the hind limbs. The rat tail was taped to a harness that was placed on a hook at the top of the cage. The hook was adjusted to allow only the forelimbs of the animal to reach the floor (30° between the floor and the body of the suspended animal) (Figure 1A). The HS animals were free to move along the cage using their forelimbs to allow locomotion and feeding (15, 32).

High-frequency electrical stimulation (HFES)

The animals were anesthetized using ketamine (75 mg/kg) and Xylazine (5 mg/kg), an incision of 2 cm was performed on the side of the right thigh. The sciatic nerve was accessed before the trifurcation; hook electrodes were placed on both sides of the nerve and the wound was sutured. After this, the rat was secured on a stimulation table and the right limb was free to perform movements (Fig. 1B). The HFES was realized with a stimulator (Grass S88 stimulator, Grass Technologies, West Warwick, RI, USA) with transformer isolation unit (RF SIU5, Grass Technologies, West Warwick, RI, USA) for the used voltage (5-24 V). Increasing voltage was applied until a visible tetanic contraction was observed (19, 21). The sciatic nerve was stimulated with pulse duration of 1 ms delivered at 100 Hz. Muscle contractions were sustained for 3 seconds, followed by 10 seconds of rest, when the limb was returned to the neutral position. This pattern of stimulation was repeated by 10 sets of 10 contractions, leaving 60 seconds of rest between each set. The protocol resulted in a total of 100 contractions per approximately 30 minutes (21).

Isolation of the soleus, tibialis anterior and extensor digitorum longus muscles

By the end of the HFES session; the soleus, TA and EDL muscles of the right hind limb (with a single session of HFES) and left limb (without HFES) of both groups were immediately isolated. Then, they

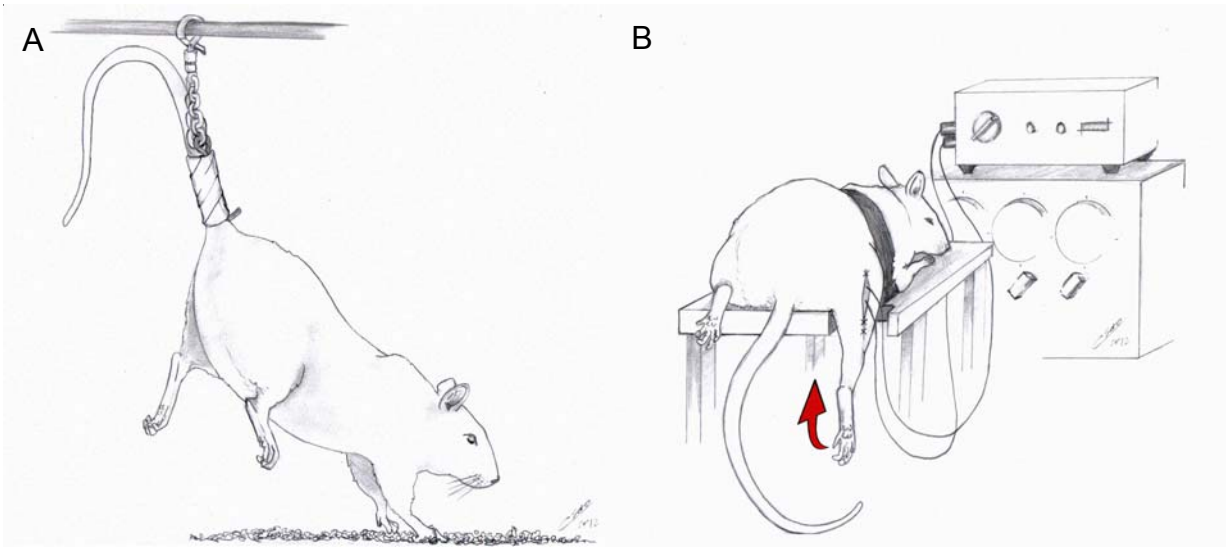

Fig. 1: A Animal model of hindlimb suspension; B Electrical stimulation protocol (only in the right limb).

were weighed and stored in a 5% formaldehyde solution, with 0.075 M sodium phosphate buffer and pH 7.3 for 24 hours at room temperature.

Evaluation of the cross-sectional area of muscles fibers (CSA-MF) of soleus, tibialis anterior and extensor digitorum longus

After fixating the samples in a phosphate-buffered 4% formaldehyde solution, a cross-section was performed on the middle portion of each muscle. Dehydration of the samples was performed through gradually immersing them in increasing concentrations of ethyl alcohol solutions. Following this, the clearance was conducted with Xylene. Paraffin was molten at 60°C and infiltrated in liquid state in the tissue while the samples were kept in an oven for 30 minutes to 1 hour at that same temperature. Then, the samples were placed in a mold and allowed to solidify at room temperature. Samples were cut into 5 µm-thick sections using a microtome (325 HM Microm, Walldorf, Germany). Finally, sections were placed on glass slides and paraffin was eliminated. Xylene was included and the sections were rehydrated with a series of washes in decreasing concentrations of ethyl alcohol solutions until a final wash in distilled water. The tissue was stained with hematoxylin and eosin (HE), then it was dehydrated again and sealed with a coverslip using a resinous mounting medium (Bio-Optica, Milano, Italy). The slides were then placed under a primo star trinocular microscope (Carl Zeiss, Jena, Germany), a cross section of muscle was selected and pictures were taken with 4-fold magnification with a camera (Canon EOS Rebel XSI, Tokyo, Japan) using the EOS Utility software (version 2.4, Copyright©, CANON INC, USA). The Adobe Photoshop CS program (version 8.0.1, Adobe System Inc., Seattle, USA) was used to organize the photographs on a whole muscle image. The total number of muscle fibers on the cross section was counted through the Micrometrics™ SE Premium software (version 2,8; ACCU-SCOPE Inc., Commack, New York, USA) and the CSA was measured using the AxioVision program (version 4.8.1.0, Carl Zeiss Imaging Solutions, Jena, Germany). The center of the cross section of the muscle was determined and guided its division into four equal quadrants. After counting the total number of fibers in the muscle CSA, the number of muscle fibers to be evaluated for CSA estimation was equally divided in these four quadrants.

Content of desmin protein in the soleus, tibialis anterior and extensor digitorum longus muscles in both groups of old rats

Samples of the soleus, TA and EDL muscles were prepared as described above. After processing, samples were cut into 5 µm-thick sections using a

microtome (325HM Microm, Walldorf, Germany). The sections were deparaffinized and hydrated using the following sequence of washes: Xylene I (15 min), Xylene II (15 min), Alcohol 100% (5 min), Alcohol 96% (5 min), Alcohol 90% (5 min), Alcohol 80% (5 min), Alcohol 60% (5 min) and distilled water (5 min). Subsequently, the antigens were unmasked by leaving the cuts in 0.01 M sodium citrate buffer, pH 6.0 for 20 min inside a steamer (Oster 5711, Florida, USA). Blocking of endogenous peroxidase was performed with a wash of 3% hydrogen peroxide for 10 min at 22°C. The slides were washed with 0.05 M Tris buffered saline (TBS), pH 7.4, 3 times for 5 min. Nonspecific blocking was performed with 3% bovine serum albumin (BSA) for 10 min at 22°C, then the excess was removed. It was added 50 µL of polyclonal rabbit primary antibody anti-desmin (D-8281, Sigma Immuno Chemicals, St. Louis, MO, USA) at a concentration of 1:50 for 12 hr at 22°C. As negative control, the incubation with the primary antibody was omitted. Then the sections were washed with 0.05 M TBS, pH 7.4, 3 times for 5 min. A final incubation was performed using the tertiary complex streptavidin peroxidase (Universal LSAB TM Kit/HRP, Rb/Mo/Goat – DAKO, Carpinteria, CA, USA), following the manufacturer's instructions. Subsequently, nuclear counterstaining was performed with Harris hematoxylin and then the samples were dehydrated, cleared and mounted using a resinous mounting medium (Bio-Optica, Milano, Italy) on the coverslips. Done all the above procedures, the slides were placed under a primo star trinocular microscope (Carl Zeiss, Jena, Germany), a cross section of muscle was elected and pictures were taken with 40-fold magnification with a camera (Canon EOS Rebel XSI, Tokyo, Japan) using the EOS Utility software (version 2.4, Copyright©, CANON INC, USA). Pictures were taken following the pattern from left to right and descending, then from right to left, without any repeated field and considering the entire CSA of the muscle. After counting the total number of fibers in the muscle CSA, the number of fibers to be evaluated for desmin content was equally divided in all the pictures. Through the program ImageJ (version 1,46j; National Institute of Health, USA), each photograph was processed using the Plug-in Colour Deconvolution. By selecting the vector H DAB and the photography Colour 2, the outline of the chosen fiber was marked. The data obtained from densitometric intensity quantification of each fiber was normalized by its respective CSA.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism® software (version 4,01; El Camino Real, CA, USA). Results are presented as mean±standard error of the mean (SEM). Data were subjected to Shapiro-Wilk normality test for data with n lower than 50 or to Kolmogorov-Smirnov test for data with a greater n. For data with a normal distribution (body weight, water and food consumption, and muscle mass) the difference between groups was analyzed by t-test. For data not normally distributed (CSA-MF and content of desmin protein) a nonparametric Kruskal-Wallis test was used with Dunns post-hoc test. The differences between groups were considered significant for $P<0.05$.

Results

Body weight and muscle mass

The initial (530.5±26.43 for C group vs 519.8±10.02 for HS group) and final (540.7±25.08 for C group; 475.0±15.01 for HS group) body weights were not significantly different between groups (Table I). It can be seen between the initial vs final body weights within each group that the C group (530.5±26.43 vs. 540.7±25.08) presented no significant differences. However, the initial vs final body weights comparison of the HS group (519.8±10.02 vs 475.0±15.01) showed was significantly lower final weight ($P=0.0031$). In considering the variation of body weight through the experiment, it can be observed an increase of 10.33±7.074 g in C group vs a decrease of 50.47±9.102 g in the HS group, which was significantly different ($P=0.0004$). There was a significant decrease in soleus ($P<0.05$), TA ($P<0.001$) and EDL ($P<0.05$) muscles mass of HS group vs C group. There were no significant differences of muscle mass after the HFES (Table I).

TABLE I: Body weight and muscle mass.

	Control group	*HS group*
Body weight		
Initial body weight (g)	530.5±26.43	519.8±10.02
Final body weight (g)	540.7±25.08	475.0±15.01
Variation of body weight after 14 days (g)	10.33±7.07	–50.47±9.10***
Soleus - Left		
Wet weight (mg)	213.3±9.88	171.7±11.38*
Ratio mg/g of b.w.	0.41±0.03	0.35±0.01
% of loss due to HS		20%
Soleus - Right (ES)		
Wet weight (mg)	201.7±6.54	168.3±10.78*
Ratio mg/g of b.w.	0.38±0.01	0.35±0.01
% of loss due to HS		17%
TA - Left		
Wet weight (mg)	1040±22.66	823.3±33.23***
Ratio mg/g of b.w.	1.98±0.06	1.73±0.04*
% of loss due to HS		21%
TA - Right (ES)		
Wet weight (mg)	1045±18.75	851.7±22.27***
Ratio mg/g of b.w.	1.99±0.06	1.79±0.03*
% of loss due to HS		19%
EDL - Left		
Wet weight (mg)	256.7±16.26	220.0±9.66
Ratio mg/g of b.w.	0.48±0.01	0.46±0.01
% of loss due to HS		14%
EDL - Right (ES)		
Wet weight (mg)	283.3±15.42	223.3±10.85*
Ratio mg/g of b.w.	0.53±0.02	0.46±0.01*
% of loss due to HS		21%

Body weight (g) and muscle wet weight (mg) of Control and HS groups of Sprague-Dawley rats with 23 months of age (n=6 per group). Muscle mass was expressed as wet weight (mg) and normalized to body weight (mg wet weight/g of b.w.). Only the right leg receives electrical stimulation. HS: hindlimb suspension; TA: tibialis anterior; EDL: *extensor digitorum longus*; ES: electrical stimulation. Values are presented as mean±SEM. Statistical test: paired t-test (comparison right vs. left) and unpaired t-test (comparison control group vs. hindlimb suspension group). **P*<0.05; ****P*<0.001.

Consumption of water, food and body weight variation

The water consumption in both groups was evaluated and the average values of 39.4 ml/day for C group and 30.8 ml/day for the HS group were obtained. Significant differences were observed only on days 7, 9 and 10 (*P*<0.05) (Fig. 2A). The food consumption in both groups was also evaluated and the average values of 25.6 g/day for the C group and 21.4 g/day for the HS group were obtained. Significant differences were only observed on days 3, 5, 8 (*P*<0.05), 10 (*P*<0.01) and 12 (*P*<0.001) (Fig. 2B). The daily variation in the body weight differs significantly between both groups during the 14 days of the study. The C group shows a trend toward weight gain without observing significant differences with respect to their initial values. The HS group has a significant body weight loss (*P*<0.05) from the day 3 until day 14 when compared with the C group. Similarly, a significant difference (*P*<0.05) was also observed from day 3 until day 14 in the HS group with respect to their initial values (Fig. 2C).

Cross-sectional area of muscle fibers (CSA-MF) of the soleus, tibialis anterior and extensor digitorum longus muscles

The HS group (without HFES) presented features of muscle atrophy, characterized by a smaller CSA-MF and larger interstitial space when compared to their respective controls (Fig. 3A). A comparison between the CSA-MF of the C vs HS groups found a significant decrease of 26.71% (*P*<0.001) in the soleus muscle; 9.81% (*P*<0.001) in TA muscle and 32.91% (*P*<0.001) in the EDL muscle of the HS group.

The comparison of CSA-MF between the C vs ES-C groups showed a significant increase (*P*<0.001) in soleus muscle and a significant decrease (*P*<0.001) in TA and EDL muscles (Fig. 3B, C, D) after HFES. Between HS vs ES-HS groups there was a significant increase (*P*<0.05) in the CSA-MF of EDL muscle (Fig. 3D) immediately after HFES.

Content of desmin protein in the soleus, tibialis anterior and extensor digitorum longus muscles

There were variations in the content and organization of the desmin protein in the studied groups, as illustrated in Figure 4A. A comparison was made between C vs HS groups, obtaining a significant decrease of 11.70% (*P*<0.001) in the soleus muscle and 8.28% (*P*<0.001) in the EDL muscle and a significant increase (*P*<0.001) equivalent to 24.70% in the TA muscle of the HS group.

The content of desmin protein between C vs ES-C groups showed a significant increase in soleus

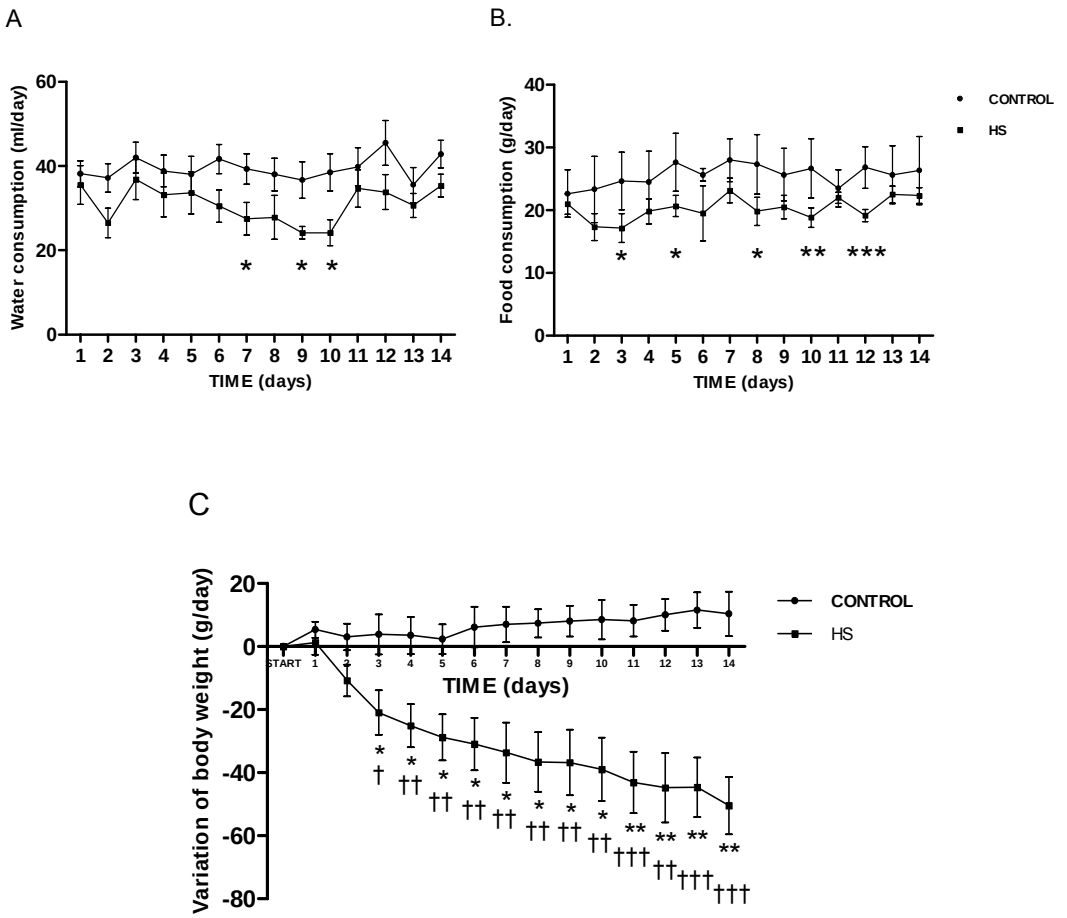

Fig. 2: Variations of (A) water consumption, (B) food consumption and (C) body weight in the 14 days of experiment. Data are expressed as mean±SEM, n=6 per group. Statistical test for (A) and (B): unpaired t-test. *P<0.05, **P<0.01, ***P<0.001 and for (C): paired t-test (within the same group); *P<0.05, **P<0.01 and unpaired t-test (control group vs. HS group) †P<0.05, ††P<0.01, †††P<0.001. HS: hindlimb suspension.

(P<0.001) and EDL (P<0.05) muscles, and a significant decrease (P<0.001) in TA muscle after HFES (Fig. 4B, C, D). Between HS vs ES-HS groups there was a significant increase (P<0.001) in soleus muscle immediately after HFES (Fig. 4B).

Discussion

Rats submitted to HS decreased their body weight, which is in accordance to that obtained by other authors (14, 16, 33). A significant decrease in wet weight was also observed in all muscles evaluated, except in the left EDL muscle (without HFES) submitted to HS. The rates of muscle mass loss were: 17% for the right soleus, 20% for the left soleus, 19% for the right TA, 21% for the left TA, 21% for the right EDL and 14% for the left EDL. The TA muscle presented the largest decrease, which may be due to having a high content of type II fibers, and these are affected to a greater extent during aging. Although, under this model of muscle disuse atrophy, muscles with prevalence of type I fibers are more affected (ex. soleus muscle) (2, 7). This interplay between the atrophic effects of aging and disuse are still being elucidated (34). Hwee and Bodine (2009), after 14 days of HS in 30 months-old male Fisher rats, observed a percentage of muscle loss of 34% for the soleus, 13% for the TA and 13% for the EDL (16), which differs from our results. This might be related to response variation in different species (Sprague-Dawley rats in the present study vs Fisher rats in Hwee and Bodine (2009) study [16]) and age (23 months-old animals in the present study vs 30 months-old in Hwee and Bodine (2009) study [16]). These same authors present a variation of body weight

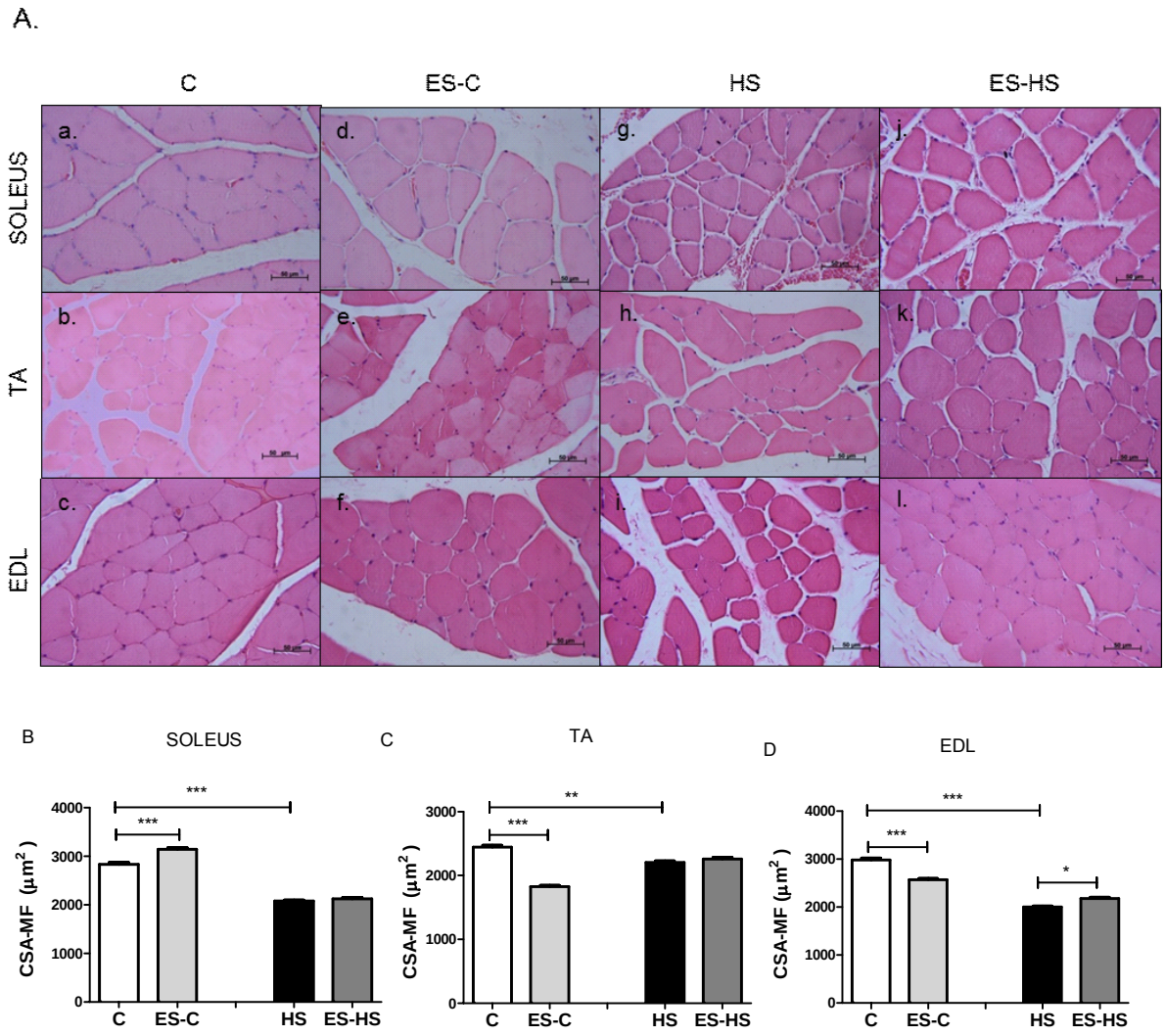

Fig. 3 : Cross sectional areas of the soleus, TA and EDL muscles fibers.

(A) Representative histological hematoxylin-eosin staining images of soleus (a,d,g,j), TA (b,e,h,k) and EDL (c,f,i,l) cross sections. Reference bar represents 50 µm. (B) (C) (D) Values are expressed as mean±SEM (n=1693-2114). Statistical test: Kruskal-Wallis and Dunn's *post-hoc* test. **P*<0.05, ***P*<0.01, ****P*<0.001. C: control group; ES-C: electrical stimulation control group, HS: hindlimb suspension group; ES-HS: electrical stimulation hindlimb suspension group; TA: tibialis anterior, EDL: *extensor digitorum longus*. CSA-MF: cross sectional area-muscle fibers.

after 14 days of HS of 3 g of weight gain for C group and a decrease of 94 g for the HS group, which approached our results (gain of 10.33 g in the C group and a decrease of 50.47 g in the HS group). When comparing the final weight percentage of variation between groups, our research found a percentage of reduction between C vs HS groups of 12.15%, similar to the results mentioned by Siu et al. (2005), in which there was a percentage of reduction of 15% due to HS for 30 months-old Fisher rats (14).

During the period of experimentation it is important to consider the consumption of water and food to assess changes in body weight, especially in a model of muscle disuse atrophy, as the drop in the values of body weight and muscle mass could be attributed to a lower intake and this may alter muscle protein metabolism in rodents (7) and humans (35). The average of water and food consumption for both groups resembles the indications for adult Sprague Dawley rats, which are 20-45 ml/day and 15-30 g/day, respectively (36). There was a slight decrease

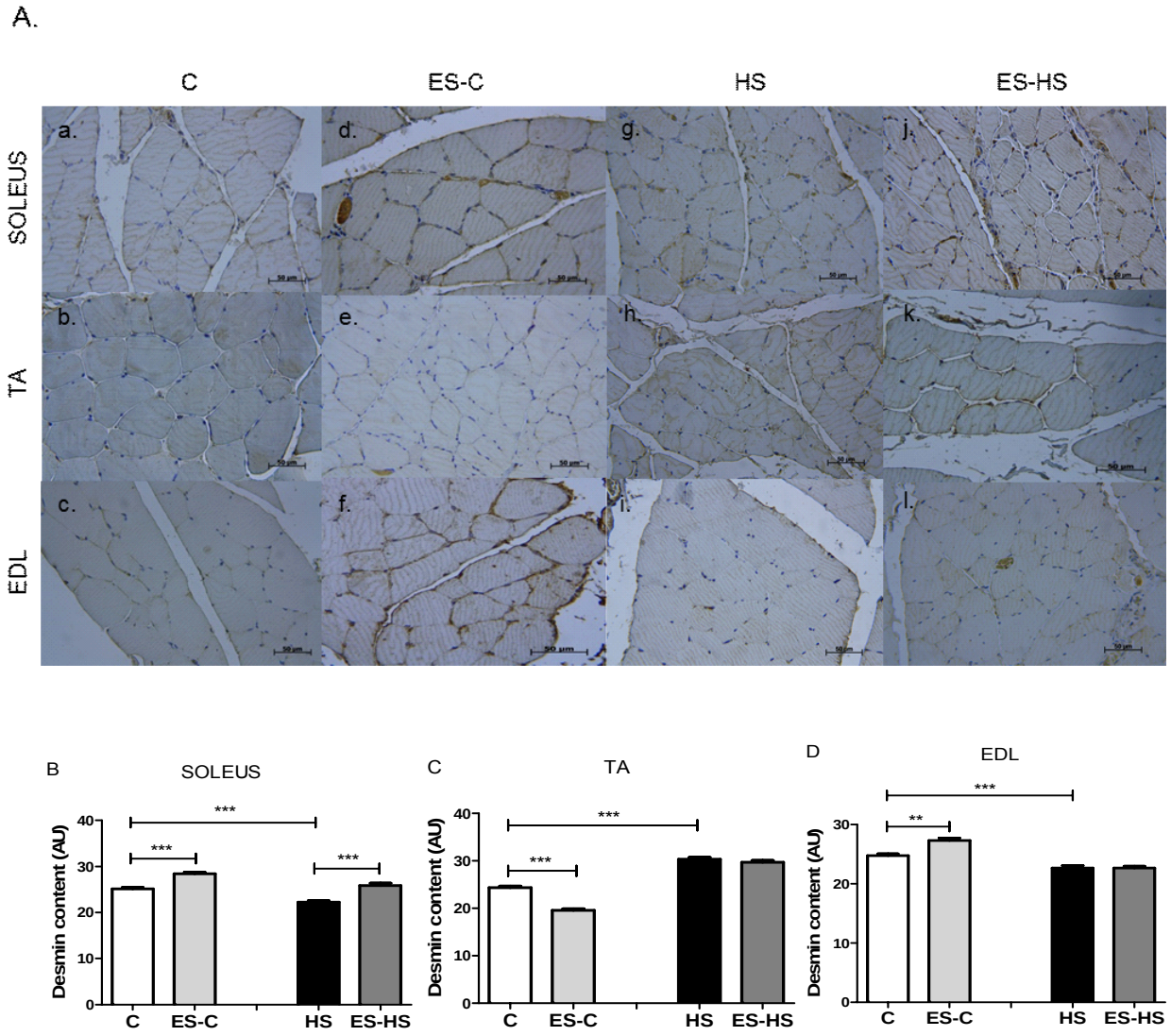

Fig. 4 : Content of desmin protein in the soleus, TA and EDL muscles fibers.

(A) Representative immunohistochemistry with diaminobenzidine (DAB) images of cross sections of the soleus (a,d,g,j), TA (b,e,h,k) and EDL (c,f,i,l) muscles fibers. Reference bar represents 50 µm. (B) (C) (D) Values are expressed as mean±SEM (n=1155-2268). Statistical test: Kruskal-Wallis and Dunn's *post-hoc* test. **P*<0.05, ***P*<0.01, ****P*<0.001. C: control group; ES-C: electrical stimulation control group, HS: hindlimb suspension group; ES-HS: electrical stimulation hindlimb suspension group; TA: tibialis anterior, EDL: *extensor digitorum longus*, AU: arbitrary units.

in food consumption during HS, which was steep in the first days, but became steady later on. This decrease in HS group may be due to the stress caused by this model (32). However, when normalizing the water/food consumption by the body mass, the HS group was not different to C group.

In this study, histological features of muscle atrophy were similar to the ones found by other authors; the HS increases the interstitial spaces (1, 2) and decreases the CSA-MF (2, 8, 37). In our results, there was a significant decrease in the CSA-MF in the three muscles studied when comparing the HS group vs C group. The HFES performed in the control group caused significant changes in CSA-MF for the three muscles (increase in the soleus and decrease in the TA and EDL muscles). With respect to soleus muscle, similar observations were obtained in studies with chronic low frequency electrical stimulation (2, 38). Muscles composed mostly by type I fibers are more able to vasodilate upon stimuli of resistance exercises, increasing extravasation due to increased

pressure caused by the concentric contraction performed. It may help to explain why an acute session of HFES immediately increased soleus muscle fiber volume (39), as the increase in the blood flow is directly related to the CSA-MF (40). We found an opposite response in the TA and EDL muscles after HFES when compared with soleus muscle. They presented a decrease in CSA-MF, which could be explained by the occurrence of damage to the muscle fibers. It may be due to the fact that muscle fibers are more susceptible to damage after eccentric muscle contraction, as observed Vijayan et al. (2001) that conducted a study of muscle fibers after eccentric exercise and found that type II fibers suffer greater harm than type I fibers after this type of exercise (41). The application of HFES produced a tetanic contraction of all muscles of the leg. The strength characteristics of the group of extensor muscles of the leg, enables the soleus muscle to contract while shortening. On the other hand, the TA and EDL muscles would contract while lengthening. Then, the decrease in CSA-MF of the TA and EDL muscles may be due to the increased damage caused by a tetanic contraction during elongation that occurs in this muscle group (41). A lengthening contraction produces a mechanical stress on the cell membrane, causing disruption in some cases, which would lead to an increase in intracellular calcium by activating calcium-activated proteases and calpains, which causes a selective hydrolysis of the intermediate filaments of the sarcomere, using as main substrate the structural proteins, such as desmin, leading to muscle fiber derangement, which can be translated as a decrease of CSA-MF (42). EDL muscle submitted to HFES showed a significant increase in the CSA-MF after a period of HS. EDL muscle of the HS group has a smaller CSA-MF, so each muscle fiber would receive a higher load during acute HFES. This, added to the repeated lengthening contractions, may cause an inflammatory process of the muscle fiber which can lead to CSA-MF increase, up to 4 times its original size (43).

Desmin protein has been studied extensively by several laboratories since its discovery in 1977 and the creation, three decades later, of the desmin knockout (KO) mice (44). Desmin KO mice studies revealed that desmin loss causes disruption in the organization of muscle fibers, reduction of active force, decreases the efficiency of power transfer and increases the susceptibility to damage due to eccentric exercise, that is to say, desmin provides protection for skeletal muscle (44-46). Furthermore, also using desmin KO mice, other authors observed that desmin protein was important in the early events of myogenesis during muscle regeneration and may have a role in the proliferation of myoblasts (47). Desmin protein loss also affects mitochondrial function in heart and skeletal muscle, which affects the performance of aerobic exercise (48). In our results, it appears that the soleus muscle tends to have higher levels of desmin, as other authors reported (30, 45). By comparing the content of desmin protein between C vs HS groups, there were significant differences with diverse response patterns. Soleus and EDL muscles have decreased content of desmin protein and TA muscle has increased desmin content after 14 days of HS. Meyer and Lieber (2012), observed a decrease in the expression of desmin protein in healthy adults subjects compared with young ones (29). On the other hand, Russ and Grandy (2011), comparing adult and old rats, observed that the content of desmin protein increases during aging in soleus, plantar and medial and lateral gastrocnemius muscles, unlike the assumption that they could be reduced (28). Ansved and Edstrom (1991), observed that the desmin was increased in some atrophic fibers and some normal size fibers of old rats compared to younger ones, possibly indicating a loss of myofilaments and an increase in the size proportion of the intermediate filaments (27). The increase of cytoplasmic desmin in muscles in denervation-reinnervation models suggests that this mechanism may also occur in aging (28). It was speculated that the increase of desmin protein content during aging is a compensation for the impaired calcium release from sarcoplasmic reticulum, suggesting that desmin protein is important for the process of excitation-contraction coupling in the sarcoplasmic reticulum and T-tubules (28, 45).

Chopard et al. (2001), observed that the relative content of desmin protein in soleus and EDL muscles does not vary after 3 and 6 weeks of HS in 6 weeks-old Sprague-Dawley rats (30). Enns et al. (2007), observed that the content of desmin protein in the

soleus and MG muscles in mice decreases in the first day of HS, is significantly reduced on the third day but is restored in the 9th day (49). In the same way, Ogneva (2010) showed that the content of desmin protein in the medial gastrocnemius and TA muscles in young rats was increased in the days 1, 3 and 7 of HS, returning almost to baseline levels on the day 12. This represents a different dynamic to the content of desmin protein in soleus muscle, on which decreases on 3 and 7 days and return to initial levels on day 12 were observed (31). This is consistent with our results: decrease of desmin content in soleus and increase in TA muscle after a period of 14 days of HS. The above studies indicate that there are discrepancies with respect to the levels of desmin protein during aging. Further research is needed to address these differences.

One session of HFES made significant changes to the content of desmin. We observed a decrease in the content of desmin protein in TA muscle and an increase in soleus and EDL muscles of the C group. This decrease in the TA muscle may be because of the HFES protocol used in this study. It causes a lot of tension in the TA muscle during elongation, what could lead to damage of the cell membrane with a consequent increase in intracellular calcium, activating calpains and phospholipase A_2 (50), which in turn use desmin protein as substrate, but not actin or myosin (42, 51). The changes of desmin protein content are involved in the early stage of injury development within the myofibril, possibly as a direct result of disruption of the cytoskeleton (42). The increase in the content of desmin protein in the soleus muscle may be because this muscle presents numerous mitochondria, and is capable of maintaining a higher ATP content during prolonged exercise. This way, the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) can maintain calcium into the sarcoplasmic reticulum, preventing the activation of calcium-dependent proteases in the cytoplasm and thereby reducing degradation of desmin protein. Lieber et al. (1996), showed in their study that injury induced by eccentric contraction was accompanied by structural changes in the EDL and TA muscles since the first 5-15 minutes of exercise. These authors speculated that the rapid changes on the state of desmin protein were not mediated by mechanisms that are based on gene regulation, because they occurred quickly. The mechanisms involved in the disruption of the muscle fiber membrane probably have enzymatic characteristics (51). Komulainen et al. (1998) showed that immediately after the elongation contractions is created a discontinuity of the edges of the fiber by disruption of dystrophin (43). Those evidences justify the prompt responses presented in this study, on which there were significant differences in desmin content immediately after a single session of HFES.

The tension generated during eccentric contraction is distributed in a smaller active CSA making structural damage in this system, which is susceptible to disruption and subsequent phagocytosis (52). Lieber et al. (1994), performing electrical simulation in rabbits for 30 minutes with frequencies from 5 Hz to 200 Hz, observed that the difference of injury in the EDL and TA muscles was consistent with the magnitude of force experienced during eccentric muscle contraction; EDL muscle showed higher percentage of desmin negative cells than TA (53). The studies with acute electrical simulation reveal that injured fibers are mostly of fast glycolytic type. For example, a type IIa fiber in the EDL muscle undergoes ~20% strain, which is more prone to injury than a type IIa fiber in the TA muscle, which undergoes only ~10% strain. In this regard, in the EDL muscle, the levels of desmin protein after HFES are reduced in a higher extent when compared to TA muscle, suggesting that the damage was higher in the EDL muscle (53). Current studies modified the initial muscle length to prevent muscle damage. Usami et al. (2011), perform electrical simulation in an initially shortened or elongated soleus muscle (50). They observed that the elongation reduces the initial injury, probably by preventing hypercontraction, which is reported to occur in soleus muscle after 30 minutes of electrical simulation (54). When making a tetanic contraction, the soleus muscle would suffer hypercontraction damage and would increase its levels of desmin protein after the electrical simulation, such as in our study. Vasaghi-Gharamaleki et al. (2011), performed acute electrical simulation in Sprague-Dawley rats with 19 to 21 weeks of age and found that after the

eccentric contraction, the fastest structural change observed in the muscle fiber was the loss of desmin protein (55). There is also controversy, as the disuse causes a decrease in the strength and CSA of soleus muscle, but does not decrease the desmin protein content after 12 days of HS (31). Further research is needed in this area.

In conclusion, a single session of HFES has different effects on the morphohistology of the soleus, TA and EDL muscles in old rats submitted to hindlimb suspension.

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