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ONE-MINUTE BOUTS OF PASSIVE STRETCHING AFTER IMMOBILIZATION INCREASE SARCOMEROGENESIS IN RAT SOLEUS MUSCLE

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ABSTRACT

Although the effect of stretching on skeletal muscle has been investigated, the mechanical influence of short bouts of passive stretch, commonly used in rehabilitation therapy to recover skeletal muscle length after immobilization, has not been studied in detail. The hypothesis of this study was that one-minute bouts of muscle stretching applied after immobilization would induce sarcomerogenesis in muscle fibers. To assess this hypothesis, sessions of passive stretching (10 stretches of 1 min each with 30 s rests between stretches) were applied daily or three times a week to the left soleus muscle after immobilization in the shortened position. Eighteen rats were immobilized for four weeks and divided into three groups: 1) after immobilization, the rats remained free for three weeks, 2) the soleus muscle was stretched daily for three weeks, and 3) the soleus muscle was stretched three times a week for three weeks. A control group was run in parallel. The cross-sectional area of the soleus muscle fibers and the serial number and length of sarcomeres were measured. Both of the stretch protocols increased the serial number of sarcomeres, but not the cross-sectional area of the muscle fibers. In conclusion, short bouts of passive stretching alter the muscle fiber tropism and induce serial sarcomerogenesis after immobilization.

Key words: Immobilization, muscle atrophy, sarcomere, skeletal muscle, stretch

INTRODUCTION

The deleterious consequences of muscle immobilization, such as the loss of serial sarcomeres [9,13,16,25,43], the proliferation of connective tissue, and muscle fiber atrophy have been extensively studied [1,43], mainly because immobilization is frequently used to treat various conditions involving skeletal muscles [8,24,26]. Although the effect of stretching on skeletal muscle has been investigated, the mechanical influence of short bouts of passive stretch, commonly used in rehabilitation therapy to recover skeletal muscle length after immobilization, has not been studied in detail.

Muscular atrophy and changes in the associated connective tissue, two of the most important adaptive alterations in muscle, involve the synthesis and degradation of muscle proteins [27]. Skeletal muscle stretching is a powerful stimulus for preventing atrophy and for inducing muscular hypertrophy [3,9,15,16]. We have recently shown that 40 min sessions of stretching applied to rat soleus muscle three times a week increased the number of serial sarcomeres and the muscle fiber cross-sectional area [9]. Similarly, 30 min of passive stretch increased expression of the myogenic differentiation-1 (myoD) and atrogin-1 genes in this muscle [17].

Models designed to investigate the effect of stretching on skeletal muscle plasticity frequently use chronic passive stretching involving immobilization with a plaster cast [30,32,41] or adhesive tape [26,41]. Although several studies have provided important contributions to our knowledge about the effect of stretching on skeletal muscle adaptability, most of them have involved long periods of muscle stretching that are not normally used in humans during rehabilitation or sports activities.

Short bouts of stretching, e.g., 0.5 - 1 min, have been recommended to treat the shortening of human muscles because of their effectiveness in improving the range of motion and flexibility [6,7]. Although sessions of short stretching are considered to be safe and effective for humans, the

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influence of this procedure on skeletal muscle has not yet been evaluated in detail in animal models. Whereas the effect of muscle immobilization and long duration stretching on skeletal muscle fiber adaptation has been studied [1,25,31,43], in the present investigation, we hypothesized that short bouts of stretching applied once a day or three times a week after muscle immobilization would be effective in inducing sarcomerogenesis in muscle fibers. The protocol used was similar to that used in rehabilitation in humans.

MATERIAL AND METHODS

Animal care and experimental groups

Twenty-four male Wistar rats (281 ± 16 g) were housed in plastic cages in a room with controlled environmental conditions and had free access to water and standard food. The rats were anesthetized with a mixture of xylazine and ketamine (12 mg/kg and 95 mg/kg, respectively, i.p.) prior to the stretching protocols and muscle dissection, and were euthanized with an overdose of anesthetics. This study was approved by the institutional Committee for Ethics in Animal Research (Protocol n° 004/03). The rats were randomly divided into fours groups (n =6/group), as follows: 1) *Immobilized and stretched three times a week (I+3StW)*: after four weeks of immobilization in the shortened position, the left soleus muscle was stretched three times a week during the following three weeks and evaluated after the last session, 2) *Immobilized and stretched daily (I+DailySt)*: after the period of immobilization, the soleus muscle was stretched daily for three weeks and evaluated after the last stretching, 3) *Immobilized (I):* after the period of immobilization, the animals remained free for three weeks prior to evaluation, and 4) *Control (C):* rats were maintained free for seven weeks.

Immobilization

To keep the soleus muscle in the fully shortened position, the left ankle joint was fixed in full plantar flexion, as previously described [9,16]. The immobilization device used was effective in producing chronic soleus muscle disuse and atrophy.

Stretching procedure

To stretch the left soleus muscle after immobilization, the left ankle was held in full dorsal flexion for 1 min (performed manually), as proposed by Ikeda *et al.* [20]. One session of passive stretch consisted of 10 stretching bouts, each maintained for 1 min, with 30 s rest intervals between bouts of stretching. Bouts of passive stretching done manually were used here because similar procedures are commonly used in rehabilitation therapy and are effective in improving flexibility in humans [11,46].

Muscle analysis

After seven weeks, all of the rats were anesthetized and weighed, and the right and left soleus muscles were carefully dissected and removed. The contralateral right soleus muscles were also examined to evaluate a possible effect of the anesthesia. The tendons were subsequently clamped with the muscle in the resting position, which was considered to be L_o, as previously described [2,9,16]. Each soleus muscle was sectioned longitudinally into two similar portions: the medial portion was used to measure the cross-sectional area of the muscle fibers, while the lateral portion was used for the sarcomere measurements.

The number and length of sarcomeres along a single muscle fiber were determined as described by Williams and Goldspink [44]. The muscle was fixed in the resting position (L_o length) for 3 h in 2.5% glutaraldehyde and then removed, placed in 30% HNO_3 for two days, and subsequently stored in 50% glycerol. Thereafter, five fibers of each soleus muscle were teased out from tendon to tendon and mounted on a slide. The muscle fiber lengths were then measured using a caliper ruler. For each fiber, the number and length of serial sarcomeres along 300 μm were quantified at different points of the mid-region using a microscope (Axiolab, Carl Zeiss, Germany). The total number and length of serial sarcomeres in each muscle fiber were determined by the correlation between the number of sarcomeres identified along 300 μm and the total fiber length, as previously reported [9,16].

For the cross-sectional area measurements, the medial portion of the soleus muscle was immediately frozen in isopentane pre-cooled in liquid nitrogen and stored at -80°C (Forma Scientific, USA). Serial cross-sections (10 μm) were then obtained from the middle belly of the frozen muscles using a cryostat microtome (Microm HE 505, Germany) and stained with 1% toluidine blue/1% borax for the morphological evaluation of muscle fibers, as previously described [35]. The cross-sectional areas of 100 muscle fibers were measured using a light microscope (Axiolab) and the Axiovision 3.6 SP4 software (Carl Zeiss). The muscle fibers were randomly chosen from the central region of a cross-section of each soleus muscle stained with toluidine blue.

Statistical analysis

The results are expressed as the mean ± standard deviation. Student´s paired *t*-test was used to compare the data for the right and left soleus muscles of the same rats in each group, and ANOVA followed by the Tukey test was used for statistical comparisons among the groups. The level of significance was set at 5% ($P<0.05$). Pearson´s

cross-correlation test was used to assess the relationship between the number and length of sarcomeres.

RESULTS

Body weight

Four weeks of immobilization greatly impaired the body weight gain compared to the control group (Table 1). Although the percentage of body weight gain was higher for the immobilized group in the remaining three weeks of treatment, the final body weight did not reach the values of the controls (Table 1).

Muscle weight and length

At the end of the experimental period (seven weeks), the right and left soleus muscles of previously immobilized rats weighed less than the control group, except for the left soleus muscle of the I+DailySt group, which was not different from control muscles (p=0.06) and showed an increase of 13.1 ± 2.8% compared with the contralateral right soleus muscle (0.21 ± 0.02 g vs. 0.18 ± 0.03 g, p=0.002) (Fig. 1A).

In general, there was no significant difference in muscle length between the right and left soleus muscles in the groups examined, except for the left soleus muscle that was stretched three times a week (I+3StW group); this muscle showed an increase in muscle length (12 ± 3%) compared to the contralateral right muscle (24.1 ± 1.3 mm vs. 21.1 ± 1.5 mm, respectively, p = 0.03) (Fig. 1B).

Muscle fiber cross-sectional area

There was no significant difference in the muscle fiber cross-sectional area (p>0.05) between right and left soleus muscle in the groups examined (I: 2181 ± 253 μm² vs. 2172 ± 335 μm², respectively; I+3StW: 2185 ± 498 μm² vs. 2567 ± 615 μm², respectively; I+DailySt: 2285 ± 236 μm² vs. 2529 ± 434 μm², respectively; C: 3723 ± 349 μm² vs. 3718 ± 609 μm², respectively), (Fig. 1C). However, there was an important decrease in the muscle fiber cross-sectional area in the right and left soleus muscles of the immobilized groups compared to the control muscles (Fig. 1C).

Serial number and length of sarcomeres

The serial sarcomere number recovered three weeks after immobilization when compared with the control muscles (Fig. 2A). However, only left soleus muscles stretched daily and three times a week after immobilization showed a significant increase in the serial number of sarcomeres when compared with the contralateral right muscles (I+DailySt: 143 ± 13 vs. 134 ± 12, respectively, p=0.007; I+3StW: 155 ± 12 vs. 138 ± 15, respectively, p=0.0001; Fig. 2A). Additionally, left soleus muscles stretched three times a week showed the highest increase in the serial number of sarcomeres (gain of 13 ± 5.6%), compared to the control and immobilized but unstretched groups (Fig. 2A).

Curiously, only muscles stretched after immobilization showed a decline in serial sarcomere length compared to the contralateral right soleus muscle (I+3StW: 2 ± 0.2 μm vs. 2.2 ± 0.3 μm, respectively; p=0.0003, decline of 10.5 ± 3%; I+DailySt: 2.1 ± 0.2 μm vs. 2.2 ± 0.3 μm, respectively, p=0.002; decline of 6.3 ± 1.4%). There was no difference in the serial sarcomere lengths of right and left soleus muscles of the immobilized, but unstretched group (2.2 ± 0.2 μm vs. 2.2 ± 0.2 μm, respectively, p=0.28) (Fig. 2B). Figure 2C shows that there was a strong negative correlation between the number and length of sarcomeres (r = -0.97).

Table 1. Body weight (g) of Wistar rats.

	Initial	4 weeks		3 weeks	
	BW (g)	BW (g)	Gain (%)	BW (g)	Gain (%)
I	278 ± 34	267 ± 33	-4 ± 11	323 ± 49†	22 ± 12
I+3StW	282 ± 17	265 ± 13	-6 ± 4	320 ± 20†	24 ± 5
I+DailySt	290 ± 33	263 ± 21	-9 ± 7	327 ± 13†	36 ± 3
C	309 ± 13	367 ± 16*	19 ± 9	421 ± 27†§	15 ± 7

The values are the mean ± standard deviation. *BW:* body weight; *I (Immobilized):* after four weeks of immobilization, the rats were allowed unrestricted movement for three weeks; *I+3StW (Immobilized and stretched three times a week):* after four weeks of immobilization, the left soleus muscle was stretched three times a week during the following three weeks; *I+DailySt (Immobilized and stretched daily):* after the period of immobilization, the soleus muscle was daily stretched for three weeks; *C (Control):* rats with unrestricted movement for seven weeks. *p<0.05: compared to the initial body weight and to the body weights of groups I, I+3StW and I+DailySt after four weeks. †p<0.05: compared to the body weight after four weeks of immobilization; §p<0.05: compared to the final body weights of groups I, I+3StW and I+DailySt. Note that the period of immobilization reduced the gain in body weight.

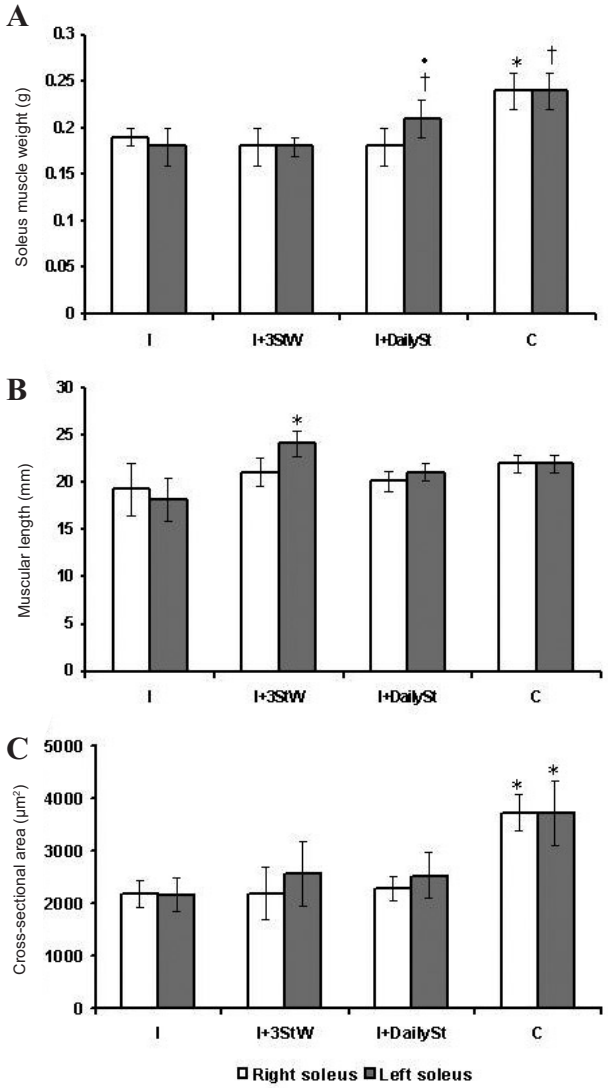

Figure 1. The effect of passive stretch on the weight, length and cross-sectional area of soleus muscle fibers in Wistar rats. *I (Immobilized):* after four weeks of immobilization, the rats were allowed unrestricted movement for three weeks; *I+3StW (Immobilized and stretched three times a week):* after four weeks of immobilization, the left soleus muscle was stretched three times a week during the following three weeks; *I+DailySt (Immobilized and stretched daily):* after the period of immobilization, the soleus muscle was stretched daily for three weeks; *C (Control):* rats were allowed unrestricted movement for seven weeks. **(A)** Soleus muscle weight (g). *p<0.05: compared to the right soleus muscle of groups I, I+3StW, and I+DailySt; †p<0.05: compared to the left soleus muscle of groups I and I+3StW; •p<0.05: compared to the contralateral right soleus muscle. **(B)** Soleus muscle length (mm). *p<0.05: compared to the contralateral right soleus and to the left soleus muscle of groups I, I+3StW and C. **(C)** Cross-sectional area of soleus muscle fibers (µm²). *p<0.05: compared to the right and left soleus muscles of groups I, I+3StW and I+DailySt. The columns are the mean ± standard deviation.

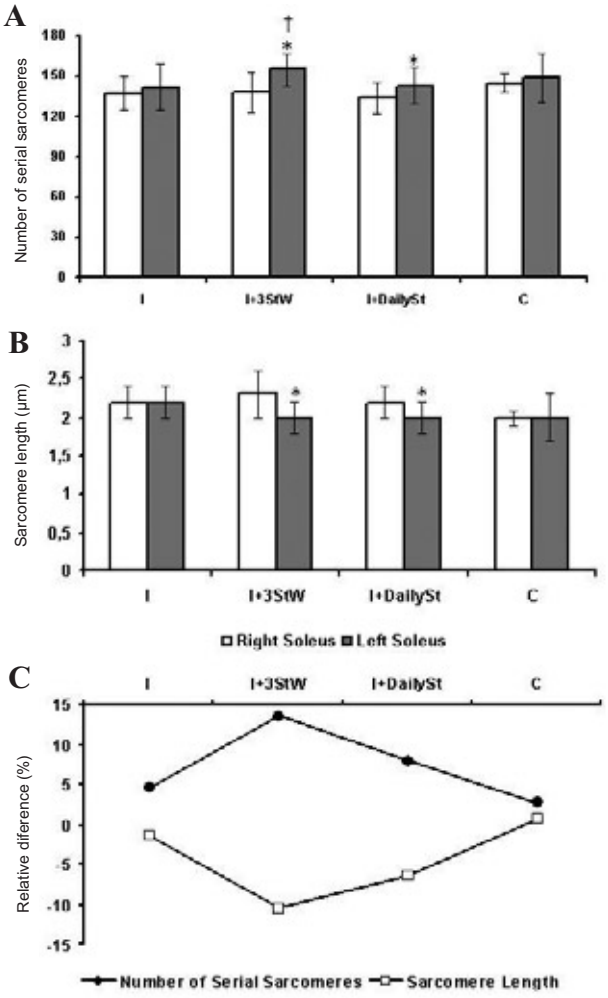

Figure 2. The effect of passive stretch on the number and length of soleus fiber sarcomeres in Wistar rats. *I (Immobilized):* after four weeks of immobilization, the rats were allowed unrestricted movement for three weeks; *I+3StW (Immobilized and stretched three times a week):* after four weeks of immobilization, the left soleus muscle was stretched three times a week during the following three weeks; *I+DailySt (Immobilized and stretched daily):* after the period of immobilization, the soleus muscle was stretched daily for three weeks; *C (Control):* rats were allowed unrestricted movement for seven weeks. **(A)** Serial number of sarcomeres. *p<0.05: compared to the contralateral right soleus muscle; †p<0.05: compared to the left soleus muscles of groups I and C. **(B)** Serial sarcomere length (µm). *p<0.05: compared to the contralateral right soleus muscle. **(C)** Pearson cross-correlation between the number of serial sarcomeres and sarcomere length (r = -0.97). The results are the mean ± standard deviation.

DISCUSSION

Our results show that sessions of passive stretching applied daily and three times a week were effective in inducing serial sarcomerogenesis in rat soleus muscle previously immobilized in the shortened position. To our knowledge, this is the first study to evaluate the effect of short bouts of passive stretching on mammalian skeletal muscle using a stretch procedure similar to that frequently used to improve the range of motion and flexibility in human rehabilitation and sporting activities.

Curiously, three sessions of stretching a week were more effective in stimulating serial sarcomerogenesis than daily sessions. Daily doses of anesthetic, as well as the stress involved in this process, could exert a negative effect on this stimulation, but this requires confirmation.

The decreased body weight seen in the immobilized rats probably resulted from the muscle disuse imposed on the rats and the consequent muscle atrophy caused by hindlimb immobilization. A similar decline in body weight has previously been reported in studies of muscle immobilization [9,41]. Factors that contribute to the weight loss associated with immobilization include a reduced food intake (resulting from the limited movement), a decrease in protein synthesis in response to hypoactivity, or some other form of stress [10,18,45].

There was a negative correlation between the sarcomere number and sarcomere length. The increase in sarcomere length in shortened muscles (group I) has been associated with a reduction in the serial sarcomere number, and probably reflects an adjustment in the sarcomere length to allow the development of maximum tension, as suggested by Stauber *et al.* [38]. In muscles immobilized in the shortened position, sarcomeres are lost and the remaining sarcomeres are pulled to a length that enables the muscle to develop its maximum tension in the immobilized position [38]. In agreement with this conclusion [39], our results showed that there was an increase in the serial sarcomere number and an equivalent decrease in the sarcomere length in the two groups that had been immobilized and then subjected to stretching. A similar relationship has been observed for sessions of passive stretching applied during the period of immobilization [9].

Despite this increase in the number of serial sarcomeres, indicative of hypertrophy, no additional gain in cross-sectional area was seen in the muscle fibers of the stretched groups compared to the group that remained free during the same period. Our results also suggest that muscle stretching differently regulated the serial sarcomere number and the cross-sectional area of the muscle fibers. Mechanical sensors that bind to the extracellular matrix of the muscle fiber cytoskeleton [19,34] can function as transducers of stretch-activated mechanical signaling and regulate gene expression in myocytes [33]. The stretching protocols used here were perhaps more efficient in activating the mechanical sensors involved in serial sarcomerogenesis than those involved in the recovery of muscle fiber cross-sectional area.

The PI3K/AKT (phosphatidylinositol-3-kinase-AKT, a serine-threonine kinase) pathway involved in the regulation of hypertrophy is activated by stretching [12]. However, the stimuli required to activate this pathway remain unknown. Ikeda *et al.* [21] showed that passive stretching of rat soleus muscle for 4 h was more efficient in stimulating myogenin expression (a myogenic transcriptional factor involved in hypertrophy) than stretching for 2 h. The authors concluded that a higher duration of passive stretching was necessary to activate these myogenic factors, and also suggested that passive stretching could be used to preserve or increase muscle strength in clinical settings. More recently, Gomes *et al.* [17] showed that daily, 30 min sessions of passive stretching increased the gene expression of myo-D in rat soleus muscle. Based on these reports, we suggest that short bouts of stretching applied daily or three times a week are able to stimulate the mechanisms associated with serial muscle fiber hypertrophy, as shown by the increase in the number of serial sarcomeres. Additional studies are required to confirm this hypothesis.

The hypertrophic effect of passive stretching has been associated with an increased muscle mass and cross-sectional areas of muscle fibers [1,4,5,9]. According to Glass [12], this hypertrophy results from the addition of myofilaments to myofibrils, thereby increasing the cross-sectional areas and the muscle strength ratio. However, none of these studies used short bouts of passive stretching similar in duration and frequency to those often used in therapeutic rehabilitation [7,12,45]. A 37% reduction in the cross-sectional area of soleus muscle fibers after four weeks of immobilization has been reported [9].

This atrophic response of skeletal muscle following muscle disuse is well known and has been described in numerous human and animal studies [2,12,14,22, 23,25,36,37,43].

The serial sarcomere number recovered completely in rats allowed unrestricted movement for three weeks after immobilization. Although the beneficial effect of gait on the recovery of serial sarcomeres was demonstrated several years ago [41], only recently has it been shown that this recovery is associated with a 45º positioning of the ankle joint, which produces maximal stretch tension in the soleus muscle during walking [39]. This angle allows the muscle to operate at an optimal length relative to the ankle joint [28,29]. These findings show that the influence of gait pattern also has to be considered when the recovery of soleus muscle is compared among mammals.

The recovery of muscle fiber cross-sectional area in rats that were allowed unrestricted movement for three weeks after immobilization probably reflected the influence of tensile strength caused by body weight on the soleus muscle during activity in the cage, thereby stimulating protein synthesis [25,36,47].

In conclusion, short bouts of passive stretching induce serial sarcomerogenesis in the previously immobilized soleus muscle of rats. Muscle stretching differently regulated the serial sarcomere number and fiber cross-sectional area. The animal model used here provides new information about the effect of passive stretching on skeletal muscle tropism after immobilization, and may have implications for rehabilitation and sport sciences. These findings require confirmation in humans although, for ethical reasons, it is difficult to examine the morphological changes in human skeletal muscle subjected to stretch.

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