ORIGINAL ARTICLE

The Effect of 30 Minutes of Passive Stretch of the Rat Soleus Muscle on the Myogenic Differentiation, Myostatin, and Atrogin-1 Gene Expressions

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ABSTRACT. Gomes AR, Soares AG, Peviani S, Nascimento RB, Moriscot AS, Salvini TF. The effect of 30 minutes of passive stretch of the rat soleus muscle on the myogenic differentiation, myostatin, and atrogin-1 gene expressions. Arch Phys Med Rehabil 2006;87:241-6.

Objective: To evaluate the effect of passive stretch, applied for 30 minutes to the rat soleus muscle, on the myogenic differentiation (myoD), myostatin, and atrogin-1 gene expressions.

Design: Case-controlled study.

Setting: University laboratory.

Animals: Fifty 12-week-old male Wistar rats.

Interventions: Six groups of animals were given a single stretch bout and were evaluated immediately and 8, 24, 48, 72, and 168 hours later. Another 3 groups were evaluated immediately after 2, 3, and 7 stretches. An intact control group was also analyzed.

Main Outcome Measures: The messenger ribonucleic acid (mRNA) levels of myoD, myostatin, and atrogin-1 were assessed by real-time polymerase chain reaction.

Results: Twenty-four hours after a single session of stretch only, the myoD mRNA levels had increased compared with the control group, whereas an increase in the atrogin-1 expression was observed after 2, 3, and 7 stretches.

Conclusions: A single session of passive stretch increased the myoD gene expression, a factor related to muscle growth. Interestingly, daily stretches increased the atrogin-1 gene expression, a gene primarily associated with muscle atrophy. The results indicated that gene expression was responsive to the number of stretch sessions.

Key Words: Atrogin-1; Rats; Muscles; MyoD protein; Stretch; Rehabilitation.

SEVERAL STUDIES[1-3] HAVE DESCRIBED the importance of stretching to prevent connective tissue proliferation, muscle fiber atrophy, and the loss of serial sarcomeres in immobilized muscles resulting in the so-called longitudinal growth. It has also been reported that stretch stimulus increases both the length and diameter of skeletal muscle.[4-6] Previous reports[7] have shown that daily sessions of passive stretch applied for 30 minutes to the rat soleus muscle, immobilized in the shortened position for 3 weeks, were enough to prevent the loss of serial sarcomeres and to maintain the joint range of motion. Also, it was recently shown that stretch sessions applied 3 times a week for 40 minutes to the soleus muscle of adult rats induced an increase in both the number of serial sarcomeres and the cross-sectional area (CSA), indicating an important hypertrophic effect.[8] The molecular mechanisms and the intracellular pathways involved in longitudinal growth are still largely unknown, although it is well established that insulin-like growth factor (IGF) and mechano growth factor are highly expressed in skeletal muscle submitted to stretch.[2,3,9,10] Also, the expression pattern of muscle-specific target genes related to stretch remains elusive.

Because hypertrophy of skeletal muscle involves an increased rate of synthesis and accumulation of proteins, an increased transcription of muscle-specific genes is therefore necessary. Nevertheless, the mechanism by which nuclei increase transcription of specific skeletal muscle messenger ribonucleic acid (mRNA) in response to a hypertrophic stimulus is not known. It has been suggested that myogenic regulatory factors (MRFs) are involved in this mechanism.[11]

MRFs are a family of skeletal muscle-specific transcription factors that control the expression of several muscle genes. The family is composed of 4 members: myogenic differentiation (myoD), myogenic factor 5, myogenin, and myogenic regulatory factor 4 (MRF4).[12] During embryogenesis, MRFs are critical in the establishment of the myogenic lineage and in controlling terminal differentiation of the myoblasts and myofibers.[12,13] It has already been reported that MRFs are upregulated in skeletal muscle hypertrophy induced by stretch.[11,14-18] The present study focused on investigating the myoD gene expression in skeletal muscle submitted to stretch, because of its role in the mechanism of muscle hypertrophy and also because MRFs respond to passive stretch according to time, age, and muscle type.[11,14,19-23] Lowe et al[11] found increased myoD gene expression in muscles maintained in a lengthened position for 6, 24, and 72 hours. Zador et al[17] also identified an increase in the myoD mRNA level after 3 days of stretching. However, no detailed description is available on the expression of MRF genes in the temporal course after a short time of stretching, for example, 30 minutes.

In addition to the myoD factor investigation, the effect of muscle stretch on myostatin, also known as growth differentiating factor 8, a member of the transforming growth factor β

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superfamily,[24] was also analyzed. There is strong experimental evidence pointing to a role of myostatin in repressing skeletal muscle growth.[24] Accordingly, increases in myostatin levels during periods of muscle inactivity have been reported,[25,26] whereas myostatin expression seems to reduce on muscle reloading.[27] Moreover, the blockage and subsequent inhibition of serum myostatin increased total body mass, muscle mass, muscle size, and absolute muscle strength.[28,29] Although, it is reasonable to assume that physical activity would probably decrease the expression of myostatin, 2 recent reports described an increase in the transcript levels of myostatin on muscles submitted to eccentric training in both rats[30] and humans.[31] However, to our knowledge, the effect of passive stretch on the myostatin gene expression in skeletal muscle has still not been investigated.

Although muscle stretch has been known as a hypertrophic stimulus,[2] curiously, some reports also describe a decrease in the CSA of the rat soleus muscle submitted to stretch.[32,33] Therefore, it is reasonable to hypothesize that trophism-related genes are regulated during stretch. Furthermore, it would be interesting to investigate the effect of stretch on genes related to atrophy.

Atrogin-1 is a gene strongly activated in atrophying muscles from different etiologies such as immobilization, hindlimb suspension, chronic renal failure, diabetes, cancer cachexia, and denervation.[34,35] Atrogin-1, also called muscle atrophy F-box protein, is an F-box protein that links the protein substrate to be ubiquitinated and degraded with the rest of the E3 and ubiquitination machinery.[36,37] So atrogin-1 can be considered as a good candidate to study possible muscle atrophy associated with passive stretch.

The models developed to investigate the effect of stretch on skeletal muscle plasticity frequently use chronic passive stretch, applying immobilization using a plaster cast.[38-40] Nevertheless, to our knowledge, only 2 articles have reported on the effect of repetitive stretch on the gene expression in muscle tissues from rats. The first showed a significant increase in the expression of myogenin in soleus muscle submitted to repetitive stretch, 15 times a minute for 4 hours,[41] and the second reported an increase in myogenin gene expression in soleus muscle submitted to repetitive stretch for 60 minutes.[42] Normally, in rehabilitation and sports medicine, muscle stretching is performed for short periods of time and its application is usually made passively, as in paralyzed and unconscious patients. Therefore, the aim of the present work was to determine the effect of daily sessions of passive stretch applied for short periods of time (30min) on the expression of 3 genes: myoD, related to muscle growth; myostatin, known as a negative regulator of muscle mass; and atrogin-1, a gene involved in muscle atrophy.

METHODS

Animal Care and Experimental Groups

We used 50 male, 3-month-old Wistar rats (weight, 373±32g). They were housed in plastic cages in a room with controlled environmental conditions and had free access to water and standard food. This study was conducted in accordance with the university approval for the care and use of laboratory animals. The rats were anesthetized by an intraperitoneal injection of xylazine (12mg/kg) and ketamine (95mg/kg) for the stretching of the soleus muscle and muscle dissection. Afterward, they were killed using an overdose of the anesthetic. To stretch the left soleus muscles, the left ankle was held in full dorsiflexion for 30 minutes by means of a piece of tape, as previously described by Williams.[1]

The animals were randomly divided into 10 groups of 5 animals each. Six groups received only a single session of stretching of the left soleus muscle for 30 consecutive minutes and were killed immediately after (n=5), and after 8 (n=5), 24 (n=5), 48 (n=5), 72 (n=5), and 168 (n=5) hours. To evaluate the effect of repetitive sessions of stretch on the soleus muscle, 3 groups of animals received daily 30-minutes session of stretch and the left soleus muscle was evaluated 24, 48, and 144 hours after the first session of stretch, and immediately after the last stretch. Thus the 24-hour group (n=5) received 2 stretches, the 48-hour group (n=5) received 3 stretches, and the 144-hour group (n=5) received 7 stretches. One group (n=5) of animals was not submitted to either procedure and the soleus was used as the control.

Each left soleus muscle of the rats was dissected, excised, and weighed. Afterward, the muscle was cut into 4 equal parts between the proximal and distal ends using a caliper. Each piece of muscle was immediately frozen in liquid nitrogen and stored at −80°C for the extraction of total RNA. Considering that there are conflicting reports in the literature regarding the distribution of the MRFs along the stretched muscle fibers,[15,17,43-45] in the present study, we used only the ends of the soleus muscle for evaluation.

RNA Isolation and Analysis

RNA was isolated from 1 frozen fragment from the distal ends of each muscle using 1mL of Trizol Reagent[a] according to the manufacturer's instructions. The extracted RNA was dissolved in Tris-Cl and ethylenediaminetetraacetic acid and quantified spectrophotometrically. The integrity of the RNA was confirmed by usual inspection of ethidium bromide stained 18S and 28S ribosomal RNA under ultraviolet light.

Reverse Transcription

One microgram of RNA was reverse transcribed using Superscript II reverse transcriptase[a] to synthesize complementary deoxyribonucleic acid (DNA). A reverse transcription reaction mixture (1μg of cellular RNA), 5× reverse transcription buffer, a deoxynucleotide triphosphates mixture containing 0.2mmol/L each of 2′-deoxyadenosine 5′-triphosphate, 2′-deoxycytidine 5′-triphosphate, and 2′-deoxyguanosine 5′-triphosphate, and 0.1mol/L of 2′-deoxythymidine 5′-triphosphate, 1μL of oligo (deoxythyminide) primer and 25U/μg of reverse transcriptase enzyme[a] were incubated at 70°C for 10 minutes and at 42°C for 60 minutes, then heated at 95°C for 10 minutes and quick-chilled on ice.

Oligonucleotide Primers

Oligonucleotide primers were designed for myostatin, atrogin, and transcription factor II D (TFIID) using the Primer Express Software.[b] Hill and Goldspink[45] described myoD and synthesized them all using Imprint. The sequences used were from mouse TFIID (forward: CCACCAACTGCTTAGCACC; reverse, GCCAAATTCGTTGTCATACC); rat myoD (forward: GGAGACATCCTCAAGCGATGC; reverse, AGCACCTGGTAAATCGGATTG); rat myostatin (forward: AGTGACGGCTCTTTGGAAGATG; reverse, AGTCAGACTCGGTAGGCATGGT), and rat atrogin-1 (forward: TACTAAGGAGCGCCATGGATACT; reverse, GTTGAATCTTCTGGAATCCAGGAT) genes.

Analysis by Real-Time Polymerase Chain Reaction

The RNA transcript levels for the different experimental and control muscles were analyzed simultaneously and the reactions carried out in duplicate in the Lightcycler (GeneAmp

5700 Sequence Detection System[b]) using fluorescent dye SYBR green detection.[b]

Statistics

Statistical analyses were performed using the 1-way analysis of variance (ANOVA) and post hoc Tukey (significance set at $P \leq .05$) to take into account the variation between the muscles of the control animals and variation between experimental groups.

RESULTS

Muscle Mass

No difference was found in the weight of the soleus muscles among any groups evaluated (table 1).

MyoD Gene Expression

An increase in the myoD gene expression was found 24 hours after a single session of stretch, when compared with the control group (3.4±0.9-fold vs 1±0.06-fold, respectively, ANOVA, $P=.001$; fig 1A). Subsequently, the myoD gene expression returned to the control levels for all the times evaluated (48h, 72h, 168h). On the other hand, when daily sessions of stretch were performed, the myoD levels remained unaltered for all periods evaluated (fig 1B).

Myostatin Gene Expression

The myostatin gene expression was not altered by any of the stretch periods tested (fig 2).

Atrogin-1 Gene Expression

The atrogin-1 gene expression was not altered after a single session of stretch, when compared with the control group (fig 3A). However, in the groups submitted to daily sessions of stretch, an increase in the atrogin-1 gene expression (ANOVA, $P \leq .05$) was found after 24 hours (2±0.4-fold; after 2 stretches), 48 hours (2.5±0.6-fold; after 3 stretches), and 144 hours (6±1-fold; after 7 stretches), when compared with the controls (fig 3B).

DISCUSSION

The results of this study show that a single session of passive stretch, performed for 30 minutes, induced a 300% increase in myoD mRNA when compared with the control soleus muscle, but did not change the gene expressions of myostatin and atrogin-1. As described in the literature, muscles immobilized in a lengthened position by a plaster cast, present an increase in the myoD gene expression.[11,16,17,45] However, in these earlier studies, the muscles were maintained immobilized in a stretched position for long periods, whereas in the present study the stretch was performed for a short period of time (30min).

A notable outcome of the present study is that a single session of stretch performed for 30 minutes was enough to induce an increase in the level of myoD mRNA, taking 24 hours to be detected, whereas in the muscles subjected to daily stretches, the myoD gene expression displayed no difference compared with the control group. These results suggest that the myoD gene expression depends on the time and also on the number of stretch stimuli applied to the muscle. Note that a single stretch increased the myoD mRNA level 24 hours later, but 2 stretches blocked this effect (see figs 1A, B, respectively). Conflicting studies have been reported regarding myoD gene expression. Previous reports using chicken muscles as the model found no increase in the myoD mRNA levels after 3, 6, 14, and 21 days of immobilization in the stretched position.[15,20] To the contrary, Eppley et al[46] observed high expression of qmf1, an avian homolog of myoD, after 3 to 16 hours in muscles submitted to a model of stretch-induced injury. Zador

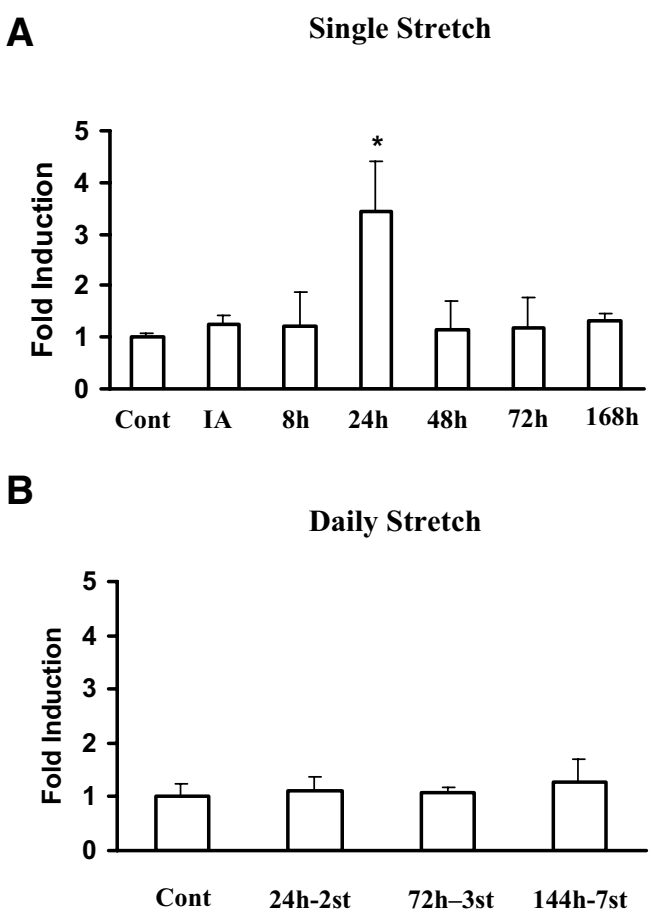

Fig 1. The effect of 30 minutes of passive stretch on the mRNA myoD levels of the rat soleus muscle. (A) Soleus muscle submitted to a single session of stretch, evaluated immediately after (IA), and 8, 24, 48, 72, and 168 hours later. (B) Soleus muscle submitted to daily session of stretch and evaluated after 24 hours (received 2 stretches [2st]), 48 hours (received 3 stretches [3st]), and 144 hours (received 7 stretches [7st]). Values are mean ± standard deviation (SD). *$P \leq .005$ (ANOVA), as compared with the control (Cont) group.

Table 1: Rat Soleus Muscle Weight

Groups	Period of Evaluation	Weight (g)
Control		.18±.03
Single stretch	Immediately after	.20±.01
	8h	.18±.02
	24h	.18±.02
	48h	.20±.02
	72h	.2±.01
	168h	.18±.02
Daily stretch	24h (2 stretches)	.17±.01
	48h (3 stretches)	.18±.01
	144h (7 stretches)	.16±.01

NOTE. Values are mean ± standard deviation (n=5 per group).

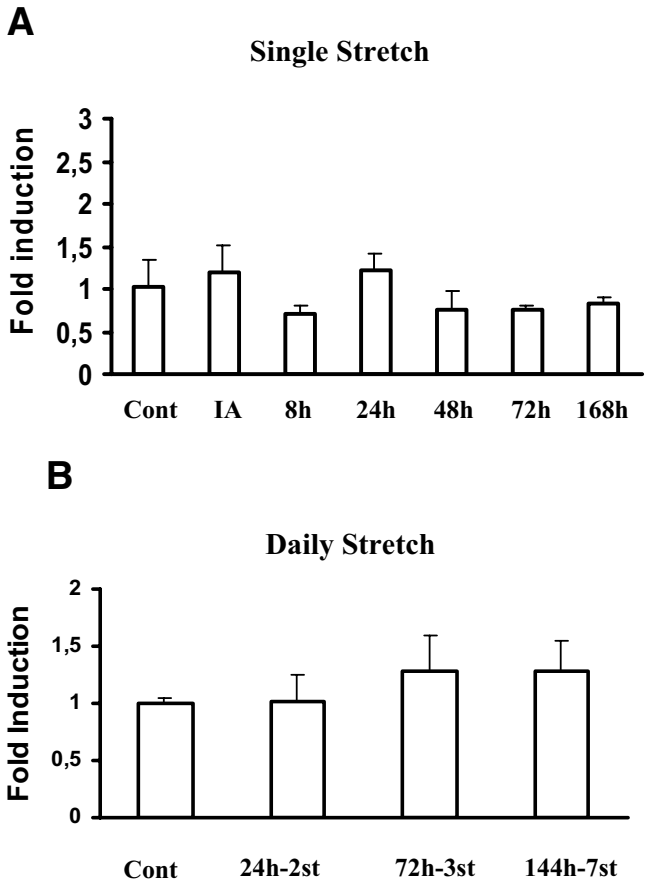

Fig 2. The effect of 30 minutes of passive stretch on the mRNA myostatin levels of the rat soleus muscle. (A) Soleus muscle submitted to a single session of stretch, evaluated immediately after (IA), and 8, 24, 48, 72, and 168 hours later. (B) Soleus muscle submitted to daily sessions of stretch and evaluated 24 hours (received 2 stretches), 48 hours (received 3 stretches), and 144 hours (received 7 stretches). Values are mean ± SD.

et al[17] also found an increase in the myoD expression of the rat soleus muscle maintained in an extended position for 3 days. Hill and Goldspink[45] also showed an increase in the myoD gene expression in the rat anterior tibialis muscle, after 1 day of immobilization in a lengthened position associated with 1 hour of electric stimulation. Peters et al[30] also reported an increase in myoD transcripts in the anterior tibialis muscle of the rat, 3 hours after a single bout of 30 eccentric contractions applied to the muscle maintained in a stretched position.

In view of the aforementioned studies, the discrepancies in myoD gene expression can be attributed to differences in the animal species, muscles, and protocols of stretch application. One possible explanation for the elevation in the myoD mRNA level may be the surge of myoD in proliferating satellite cells early after stretch. Satellite cell proliferation typically begins 24 to 48 hours after regeneration and during the subsequent events, MRFs are still until expressed in these cells.[47] Mononuclear cells with myoD peak expression were still described after 24 hours.[47] However, a previous study showed that the myoD mRNA level was elevated even in stretched-overloaded muscles irradiated for the elimination of satellite cell proliferation, presenting evidence that myoD activation is not dependent on satellite cell proliferation.[16]

IGF-1 is a protein growth factor that can induce skeletal muscle hypertrophy by activating the phosphatidylinositol 3-kinase (PI3K)–serine/threonine kinase (Akt) pathway.[48] It has been shown that the interaction between Rho, a guanosine triphosphate–binding protein with guanosine triphosphatase activities, and the serum response factor (SRF), a DNA-binding protein, is required for the regulatory pathway that controls the myoD gene expression, and that the Rho/SRF activities are dependent on IGF factors.[44,49,50] Thus it is tempting to speculate that if IGF is related to hypertrophy and myoD activation depends on Rho/SRF activation, the data found in this work could mean that the increase in myoD mRNA level induced by a single session of passive stretch for 30 minutes was enough to produce a hypertrophic signal. Moreover, it was recently reported that the induction of the IGF-1/PI3K/Akt pathway prevents the induction of requisite atrophy mediators such as atrogin.[51] This statement corroborated the present results because myoD and atrogin-1 were never concomitantly upregulated in the stretched soleus muscles.

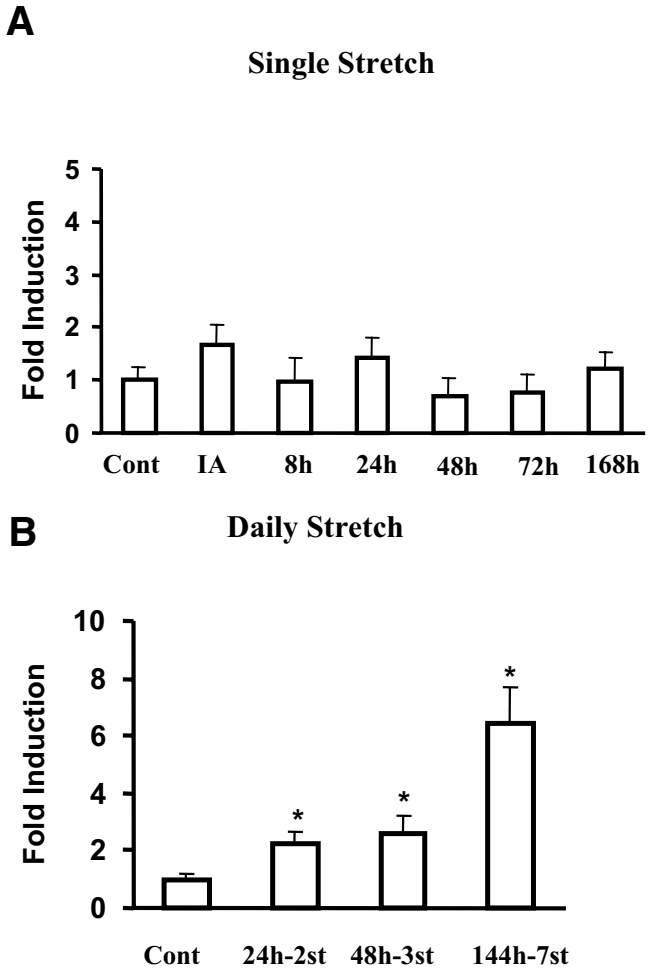

Fig 3. The effect of 30 minutes of passive stretch on the mRNA atrogin-1 levels of the rat soleus muscle. (A) Soleus muscle submitted to a single session of stretch, evaluated immediately after (IA), and 8, 24, 48, 72, and 168 hours later. (B) Soleus muscle submitted to daily sessions of stretch and evaluated after 24 hours (received 2 stretches), 48 hours (received 3 stretches), and 144 hours (received 7 stretches). Values are mean ± SD. *$P \leq .005$ (ANOVA), as compared with the control group.

The present results with myostatin show that its gene expression was not changed either after a single session of passive stretch or when the stretch session was performed daily, which indicates that passive stretches applied for a short period of time did not alter the myostatin gene expression. Recently it was also reported that eccentric exercise induced an upregulation in the myostatin mRNA expression[30,31] whereas concentric training reduced its expression.[52] These findings suggest that the level of stress in the muscle fibers could be a determinant factor in the responsiveness of the myostatin gene expression.

The data on the atrogin-1 mRNA levels showed no changes after a single session of stretch but an increase in the atrogin-1 gene expression was found when daily bouts of stretch were performed. To our knowledge, this is the first report on the effect of stretch on the atrogin-1 gene expression. Previous studies have described a decrease in the muscle fiber CSA after stretch in the rat soleus muscle,[32,33] suggesting an involvement of cellular degradation pathways. The results of the present study confirm this hypothesis because the atrogin-1 gene expression was upregulated after daily stretches.

Overall, the present study provides new evidence on the effect of passive stretch for short periods of time in the expression of genes related to skeletal muscle hypertrophy and atrophy. Furthermore, it would be interesting for rehabilitation and sports medicine if similar procedures were also evaluated in human muscles.

CONCLUSIONS

A single session of passive stretch for 30 minutes increased myoD gene expression, suggesting a hypertrophic effect. However, daily sessions of stretch blocked this effect and increased atrogin-1 gene expression, which has been primarily associated with muscle atrophy. Thus, passive stretch alters both hypertrophic and atrophic skeletal muscle mechanisms. Also, the number of stretch sessions was determinant in both atrogin-1 and myoD responsiveness.

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Suppliers

a. Invitrogen Corp, 1600 Faraday Ave, PO Box 6482, Carlsbad, CA 92008.
b. Applied Biosystems, 850 Lincoln Centre Dr, Foster City, CA 94404.