THE EFFECTS OF A SINGLE BOUT OF RESISTANCE EXERCISE ON SERUM LEVEL OF MYOSTATIN AND FOLLISTATIN IN ELDERLY MEN

*Seyed Hadi Seyedi and Mohammad Reza Ramezanpour
Department of Physical Education and Sport Sciences, Mashhad Branch, Islamic Azad University, Mashhad, Iran
*Author for Correspondence

ABSTRACT
Aging is associated with progressive loss of strength and muscle mass that this process is commonly referred to as “sarcopenia”. Decline in muscle mass and strength related to aging or sarcopenia leads to significant impairment in the ability to carry out normal daily functions and an increased risk of falls and fractures. Muscle hypertrophy is likely to result from the cumulative effects of repeated bouts of resistance exercise on post-exercise molecular responses. Therefore, we determined muscle growth-and regeneration-related factor in response to a single resistance exercise bout in elderly men. Twelve healthy elderly men (age 64.2±3.2 years, weight 76.6±6.5 kg, height 177.5±4.8 cm, fat mass 18.2±2.6 kg, lean body mass 57.2±4.7 kg) volunteered as subjects and performed one bout of lower body resistance exercise consisted of 3 sets of 12 repetitions at 75% 1-RM. Blood samples were obtained at rest and immediately, 4hr and 24hr after resistance exercise bout. Serum levels of myostatin and follistatin were determined by ELISA and Data were analyzed with repeated-measure ANOVA. Serum level of myostatin was significantly decreased (P<0.05) immediate and 4h after resistance exercise (13.34±4 and 12.68±4.3ng/ml, respectively) compared to pre-exercise (17.22±5.2ng/ml). Conversely, the single resistance exercise bout increased (P<0.05) serum level of follistatin in 4h and 24h after resistance exercise (13.2±2.4 and 13.09±2.1ng/ml, respectively) compared to pre-exercise (8.79±2.8ng/ml). These results suggested that a resistance exercise in older men by decreased serum level of myostatin and increased serum level of follistatin can prevented of sarcopenia. These changes may reveal the molecular mechanisms involved in the muscle hypertrophy reported in the elderly men after resistance exercise. This is possible eventually caused reduce the incidence of age-related muscle atrophy (sarcopenia) in the elderly.

Keyword: Myostatin, Follistatin, Elderly Men, Resistance Exercise

INTRODUCTION
Aging is related to a gradual loss of skeletal muscle mass, strength, and power, a condition called sarcopenia. Consequently, older people are predisposed to different disabilities, increased incidence of falls, and decreased capacity to perform daily tasks (Bodine et al., 2001). Importantly, in older adults, repeated resistance exercise has well-established beneficial effects on body composition and physical fitness (Borst et al., 2002; Carlson et al., 1999) and on the capacity to perform daily tasks of living (Carlson et al., 1999). These training adaptations are likely to result from the cumulative effects of repeated bouts of resistance exercise on post-exercise molecular responses (Cesari et al., 2008). During the past years, the underlying mechanisms responsible for muscle mass wasting related-aging have been extensively investigated (Carlson et al., 1999; Cesari et al., 2008). One of the hypothetical pathways by which repeated resistance exercise induced short-term molecular responses may eventually lead to muscle hypertrophy involves gene regulation via the myostatin pathway (Craig et al., 2008). Myostatin (also known as growth/differentiation factor-8) is a member of the transforming growth factor-β family that plays an essential role in the regulation of skeletal muscle mass (Gilson et al., 2009; Glass, 2005). Strength training induced muscle hypertrophy involves satellite cell proliferation, differentiation, and fusion with existing myofibers, which is important for the maintenance of adequate nuclear/cytoplasmic ratio during muscle growth (Bodine et al., 2001). Myostatin seems to work in adult muscle in part by
Research Article

inhibiting satellite cell proliferation and differentiation (Gonzalez-cadavid et al., 1998). Myostatin probably acts in this process by down-regulating the family members of myogenic regulatory factors MyoD and myogenin (Gotshalk et al., 1997) as well as cyclins and cyclin-dependent kinases (cdk) and by increasing the expressions of cdk inhibitor p21 and, possibly, also p27 (Hill et al., 2002; Hughes et al., 2002), all these affecting satellite cell cycle regulation (Gotshalk et al., 1997; Hughes et al., 2002). Myostatin mediates its actions through binding to activin IIB receptors (Hill et al., 2002). Serum myostatin was also inversely correlated with total body muscle mass in relation to height in older versus younger adult males and females (Jespersen et al., 2011; Joulia et al., 2003). Elevated circulating levels of myostatin have also been associated with muscle atrophy due to prolonged bed rest (Kawada et al., 2001). In contrast, follistatin is a glycosylated plasma protein, which is a member of the TGF-β superfamily. Follistatin has been demonstrated to bind other members of the TGF-β superfamily, including myostatin (Kraemer et al., 2002). Follistatin prevents myostatin from binding to the activin IIB receptor, by binding and thereby neutralizing myostatin in the circulation (Kraemer et al., 1990). Because myostatin is a potent negative regulator of muscle growth, the inhibition of myostatin results in marked increases in muscle mass (Lalani et al., 2000). Follistatin over-expression promotes muscle hypertrophy by satellite cell activation and is caused in part, by inhibition of myostatin (Langley et al., 2002). Thus If sarcopenia is indeed an important risk factor for clinical outcomes (especially physical disability) in older persons, interventions aimed at preventing/delaying/reducing its onset and/or progression are needed (Laurentino et al., 2012). To date, the most promising data in this context are reported from clinical trials on physical exercise, especially resistance training. In fact, significant improvements in muscle strength and physical performance have been observed after physical exercise, even among very frail older persons. As of late, research has begun to explore the expression profiles of myostatin in regard to resistance exercise. Employing dynamic muscle actions utilizing a pneumatic lower-body resistance exercise device in humans, Roth et al., (2003) showed decreases in myostatin mRNA expression after 8wks of lower-body resistance training (Laurentino et al., 2012). However, in rodents Peters et al., (2003) showed increases in myostatin mRNA expression after a single exercise bout involving only eccentric muscle actions (Lee and McPherron, 2001). More recently it has been shown that 12wks of dynamic lower-body heavy resistance training with primarily free-weight training exercises increased skeletal muscle myostatin mRNA and protein, along with serum myostatin and follistatin (inhibits myostatin binding with activin IIB receptor) (Lee, 2007). Regarding the disparity of results between different study and because of their similar opposite-acting influence, we considered whether inverse changes in the circulating levels of these factors would occur in responses to a single bout of resistance exercise. Secondly, it is known that the amount of muscle mass used and the volume (intensity, sets, and repetitions) of resistance exercise performed affects the extent of acute circulating endocrine and metabolic responses (Ma et al., 2003; McPherron et al., 1997; Patel and Amthor, 2005). So the purpose of this study was to determine the effects of a single bout of resistance exercise on serum levels of myostatin and follistatin in elderly men.

MATERIALS AND METHODS

Subjects

Twelve untrained, recreationally active males were recruited to participate in the study. The subjects were untrained from the standpoint that they had not engaged in consistent weight training for 3 months. Prior to the study; however, all were recreationally active. The twelve subjects had a mean age of 64.2 ± 3.2 years, height of 177.5 ± 4.8 cm, and body mass of 76.6 ± 6.5 kg. Before participating each subject completed a medical history questionnaire, was informed of the experimental protocol, and signed a university approved informed consent form. Subjects with contraindications to exercise as indicated by the American College of Sports Medicine (ACSM, 2000) were not allowed to participate. Body mass was measured to the nearest 0.5kg (Seca Beam Balance 710) with subjects lightly dressed and bare footed. Standing height was measured to the nearest 0.5cm (Seca Stadiometer 208). Percentage body fat, fat mass and lean body mass were calculated according to published guidelines (American College of Sports Medicine 2000), i.e. from seven skinfold measures
(average of two measurements of each site) using a Harpenden (John Bull, UK) calliper. The subjects’ physical characteristics are given in Table 1.

Experimental Design

The experimental protocol consisted of two to three familiarization sessions with the training device, followed by a one-repetition maximum (1-RM) assessment and finally a single resistance exercise bout. On the day of the experimental trial the resistance exercise was performed in the morning, and the subjects were fasted overnight before the exercise session. A baseline venous blood samples were taken from the antecubital vein after 30 min of supine rest. The subjects then performed a 10-min light warm-up on a treadmill followed by the 3 sets of 12 repetitions at 75% of 1 RM on lower body muscles. A second blood samples were taken from the opposite side in immediately, 4h and 24h post-exercise.

Exercise Protocol

The resistance exercise bout started with a 10-min warm-up on a treadmill. All subjects completed the exercise protocol consisting of five lower body exercise (squat, reverse hack squat, leg press, leg extension and leg curl) with 3 sets of 12 repetitions. The intensity of exercise was 75% 1-RM. Each repetition was approximately 2-3 seconds in duration, each repetition was separated by 45-Sec rest interval, and each set were separated by a 90-S rest interval (Peters et al., 2003). All exercises were performed using TechnoGym (Italy) equipment.

Determining 1-RM

Prior to 1-RM assessments, a familiarization session was conducted in order to minimize learning effects on the strength testing protocols. In these sessions specific exercise techniques were tough and sub-maximal practice for each exercise session determined. The 1-RM for each exercise reached within five attempts, after a brief warm-up of five to eight repetitions with loads of approximately 50% of the anticipated 1-RM. During the 1-RM process, the attempts were separated by 3–5 min breaks. All strength assessments (pre- and post-testing sessions) followed the same direction, and each test was followed by an adequate recovery period of at least 10 min (Lee, 2007). All lifts were performed according to standardized procedures and were monitored by the staff.

Blood Collection and Analysis

Venous blood samples consisted of approximately 10 ml of blood drawn from the antecubital vein using a vacutainer apparatus 48h prior to exercise bout and at immediate, 4h, and 24h after the exercise bout. Blood was centrifuged at 30001 g for 30 min and serum was extracted and then stored at a temperature of -80°C.

Table 1: Physical characteristics of subject’s

<table>
<thead>
<tr>
<th>Age</th>
<th>64.2±3.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height(cm)</td>
<td>177.5±4.8</td>
</tr>
<tr>
<td>Weight(kg)</td>
<td>76.6±6.5</td>
</tr>
<tr>
<td>Body mass index</td>
<td>24.3±3.3</td>
</tr>
<tr>
<td>Fat mass(kg)</td>
<td>18.2±2.6</td>
</tr>
<tr>
<td>Lean body mass(kg)</td>
<td>57.2±4.7</td>
</tr>
</tbody>
</table>

Myostatin Assessment

Serum myostatin concentrations were assessed using a competitive enzyme linked immunosorbent assay (ELISA) method. At first, the wells of microtiter plates were coated with recombinant human myostatin (R&D, DGDF80) (300ng/ml) dissolved in phosphate-buffered saline (PBS, pH 7.2, 0.01M) for 12 h at room temperature. The plates were washed four times with PBS. The wells were blocked with 300ul of 3% bovine serum albumin (BSA) in PBS to prevent non-specific binding and the plate incubated for 1 h. Sera were mixed with equal volume of mouse anti-human myostatin monoclonal antibody (500ng/ml) in PBS containing 0.1% BSA for 1 h. Standard curves were constructed by mixing serial dilutions of recombinant human myostatin with equal volume of the same anti-human myostatin antibody (500ng/ml) in PBS containing 0.1% BSA for 1 h. The mixtures were transferred to the coated well and incubated for
Research Article

1 h at room temperature. After washing the wells with PBS containing 0.05% Tween 20 (PBST), peroxidase-conjugated rabbit anti-mouse polyclonal antibody was added to the wells and the plate was incubated for 1 h at room temperature. Finally, it was washed six times with PBST and then 100 ul of 3,3,5,5 tetramethylbenzidine (TMB) reagent was added and incubated at room temperature for about 10 min.

Peroxidase reaction was stopped by adding 100ul of 200mM H2SO4, and the optical density at 450nm was determined by a microplate reader. All the standards and samples were assayed in duplicate. The data for the standard curve were fitted to a logistic plot and the levels of myostatin were calculated.

**Follistatin Assessment**

Follistatin concentrations in serum were determined using a sandwich ELISA. At first, 100ml of the mouse anti-human follistatin monoclonal antibody (R&D, DFN00), dissolved in PBS was added into the wells of the microtiter plate and incubated for 12 h at room temperature. Assay plates were washed three times with PBS and blocked with 300ul of 3% BSA in PBS to prevent non-specific binding and incubated for one more hour.

Then the sera were diluted three times with PBS containing 0.1% BSA and 100ul of diluted samples were added into the wells and incubated for 1 h at room temperature. Serial dilutions of recombinant human follistatin in PBS containing 0.1% BSA were prepared and added into the wells and incubated for 1 h at room temperature to construct a standard curve. Thereafter the wells were washed four times with 400ul PBST.

Then, 100ul of the goat anti-human follistatin polyclonal antibody (400ng/ml) diluted in PBS containing 0.1% BSA, were added to the wells, and the plates were incubated for 1 h at room temperature. They were washed four times with 400ul PBST, and subsequently 100ul of HRP-conjugated rabbit anti-goat antibody diluted with PBS containing 0.1% BSA was added. The plates were incubated for 1 h and washed six times with PBST.

Then, 100ul of TMB reagent was added to the wells. The reaction was stopped by adding 100ul of 200mM H2SO4, and color intensity was measured at 450nm. All measurements were performed in duplicate and data for the standard curve were fitted to a logistic plot and the levels of follistatin calculated.

**Statistical Analysis**

All data are expressed as mean values ± standard deviation. Data normality and variance equality were assessed through the K-S and Levene tests respectively. A repeated measure ANOVA with Dunn’s post hoc test was performed. Statistical significance was accepted at $P \leq 0.05$.

**RESULTS AND DISCUSSION**

Serum level of myostatin was reduced significantly ($P<0.05$) at immediately and 4h post-exercise compared with the pre-exercise. The result also indicated that serum myostatin increased at 24h after exercise, but did not achieve significant difference from pre-exercise values.

Serum of follistatin was elevated at immediately post-exercise compared with the Pre-exercise but difference not significant. In addition, serum level of follistatin was elevated significantly ($P<0.05$) at 4h and 24h post-exercise respectively compared with the pre-exercise.

**Table 2:** Mean (±SD) data for selected dependent variables in response to single bout of resistance exercise for the three times of recovery

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pre-exercise</th>
<th>Post-exercise</th>
<th>4hr</th>
<th>24hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myostatin (ng/ml)</td>
<td>17.22±5.2</td>
<td>13.34±4.1†</td>
<td>12.68±4.3†</td>
<td>19.04±3.1†</td>
</tr>
<tr>
<td>Follistatin (ng/ml)</td>
<td>8.79±2.8</td>
<td>10.37±2.4</td>
<td>13.2±2.4†</td>
<td>13.09±2.1‡</td>
</tr>
</tbody>
</table>

* Significantly ($p < 0.05$) different at immediately post-exercise
† Significantly ($p< 0.05$) different at 4hr post-exercise
‡ Significantly ($p< 0.05$) different at 24hr post-exercise
Discussion
The aim of this study was investigated the effects of a single bout of resistance exercise on serum level of myostatin and follistatin in elderly men. The results from the present study demonstrate that serum level of myostatin at immediately and 4hr after resistance exercise were significant decreased in comparison to pre-exercise. This finding was agreement with Roth et al., (2003) and Ryan et al., (2010) who reported decreased myostatin serum after resistance exercise (Laurentino et al., 2012). In contrast, Willoughby (2004) demonstrated increases in serum myostatin following heavy resistance exercise (Lee, 2007). These inconsistent findings may be due to differences in subjects, blood sampling time, exercise mode, intensity, and duration making comparisons between the studies difficult.

Myostatin is well established as a negative regulator of muscle growth in animal models (Lee and McPherron 2001). The mechanism by which myostatin inhibits muscle growth is uncertain, although (Carlson et al., 1999) (Peters et al., 2003) and others (Langley et al., 2002; Laurentino et al., 2012) have suggested an inhibitory effect on skeletal muscle satellite cells. We pursued the present investigation with the hypothesis that myostatin mRNA levels would be reduced in response to a program of heavy-resistance ST designed to increase muscle mass and strength (i.e., inhibition of the growth inhibitor). (Wehling et al., 2000) (Rios et al., 2002) demonstrated that periodic reloading of otherwise unloaded rat plantaris muscle resulted in less elevated myostatin levels (55% increases versus ambulatory controls) compared with a continuously unloaded condition (110% increase versus ambulatory controls). Moreover, Lalani et al., (2000) reported increased myostatin mRNA and protein in several rat muscles in response to microgravity, and a normalization of those levels after the return to normal gravitation (Rosenberg, 1989). Recent work by Kawada et al., (2001) demonstrated that myostatin expression was significantly reduced upon reloading of hindlimb suspended muscle in mice, with no change in expression in response to either aging-related or unloading-induced atrophy (Roth et al., 2003). These data indicate that myostatin is responsive to muscle loading in some contexts, and thus might represent an important cell size regulator for skeletal muscle. Our results indicate that a single bout of resistance exercise leads to a significant reduction in serum of myostatin in older men, which is consistent with previous observations in animal models (Gonzalez-cadavid et al., 1998; Lalani et al., 2000).

Saremi et al., (2010) shows that 12 week resistance training (hypertrophic model) decreases serum myostatin (Routh et al., 2003). However, the myostatin gene response over a period of resistance training is still controversial. For example, Roth et al., (2003) observed a decrease in myostatin gene expression after 9wk of resistance training (Laurentino et al., 2012). Conversely, Willoughby (2004) demonstrated that 12wk of resistance training resulted in increased myostatin mRNA expression, myostatin protein content, and myostatin serum levels (Lee, 2007). It may be speculated that these dissonant findings are related to the intensity of the exercise. Roth et al., (2003) performed resistance exercise with hypertrophic model (75% 1-RM), whereas Willoughby (2004) uses of heavy resistance exercise model (85-90% 1-
Exercise. These changes may reveal the molecular mechanisms involved in the muscle hypertrophy and can prevent muscle atrophy in vivo. Bod et al., 2002). The results of this study demonstrate that serum level of follistatin was a significant increase at 4hr and 24hr after exercise. These changes may reveal the molecular mechanisms involved in the muscle hypertrophy reported in the elderly men following resistance training. Since serum myostatin is inhibited from binding to the activin IIb receptor by follistatin (Wehling et al., 2000), follistatin likely plays a role in reducing myostatin signaling within skeletal muscle. Therefore, based on present study and previous results (Lee, 2007), we submit that in elderly, apparently healthy males participating in resistance exercise the increases in serum follistatin that accompany increased serum myostatin may serve to inhibit myostatin signaling and muscle catabolism that could conceivably accompany heavy resistance exercise.

Many binding proteins for myostatin have been described, but their specific role in regulating myostatin activation and receptor binding is not clear (Wehling et al., 2000). One of the binding proteins and inhibitors of myostatin is follistatin. It has been shown to be expressed in human skeletal muscle, circulates in human blood, and inhibits myostatin activity (Peters et al., 2003). We observed an increase in follistatin serum at 4h and 24h after exercise. To our knowledge, our results are the first reported findings on the behavior of the follistatin response after resistance exercise in elderly men. It is likely that the increased follistatin may have inhibited myostatin from binding to the activin IIb receptor. This is conceivable because serum myostatin is bound to, inhibited, and negatively regulated by follistatin (Peters et al., 2003).

In the present study, the reduction in serum myostatin was followed by an increase in serum follistatin, which are endogenous inhibitors of activin and other TGF-β superfamily members, including myostatin (Jespersen et al., 2011). Previous studies have also demonstrated that the follistatin mRNA level is up-regulated during muscle regeneration and muscle growth (Peters et al., 2003) whereas myostatin mRNA level decreases concomitantly.

More recently, Laurentino et al., (2012) reported that resistance training promoted a concomitant decrease in myostatin and the increase in follistatin isoforms, GASP-1 mRNA gene expression in healthy elderly men (Willoughby, 2004).

**Conclusions**

In conclusion, the present study showed specifically that a single bout of resistance exercise can down-regulated myostatin and up-regulated follistatin in older men. So resistance training is the most effective and safe intervention to attenuate or recover some of the loss of muscle mass and strength that accompanies aging.

The molecular mechanisms of skeletal muscle hypertrophy have considerable clinical importance and understanding these mechanisms may help individuals suffering from muscle wasting conditions due to age or illness, or perhaps even healthy athletes with a desire to enhance muscle strength and mass. Further work is necessary to elucidate the significance of these muscle regulators and their association with skeletal muscle hypertrophy.

**REFERENCES**


Research Article


Research Article


Zhu X, Topouzis S, Liang LF and Stotish RL (2004). Myostatin signaling through Smad2, Smad3 and Smad4 is regulated by the inhibitory Smad7 by a negative feedback mechanism. Cytokine 26 262–272.