IMPACT OF REPEATED BOUTS OF ECCENTRIC EXERCISE ON SKELETAL MUSCLE MORPHOLOGY AND MYOGENIC GENE EXPRESSION

Outlined Booklet of the PhD Thesis

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Budapest
2009

I. INDRODUCTION

Exercise-induced muscle damage is classified as a type I muscle strain injury and presents with tenderness or stiffness to palpation and/or movement. Its noticeable symptoms include, among others, Delayed Onset Muscle Soreness (DOMS), strength deficit, decreased range of motion, muscle swelling and therefore is a phenomenon that that commonly excruciates athletes/individuals.

In the last decades, although, a remarkable effort has been made to understand this particular phenomenon a lot of issues remain to be elucidated. Particularly, there is little information regarding the continuation of exercise with damaged muscles. Evidence suggests that exercise continuation prior recovery do not exacerbate muscle damage and neither affects the ability of skeletal muscle to recover. This evidence, however, have been based on indirect markers of muscle damage the reliability of which have been challenged by several studies. Consequently, this issue to be resolved requires morphological evidence and direct quantification of muscle damage. Furthermore, there are no data, regarding the effects of exercise continuation prior full recovery, on other physiological aspects.

It is well known that once skeletal muscle is damaged, as a result of an unaccustomed eccentric exercise, requires approximately 10 days or more to recover. In practical situations, however, the resting interval needed for the skeletal muscle to recover is not always available because most of the training plans are performed more than once per week. Consequently, there is an emergent need to determine the effects and subsequently the appropriateness of exercise continuation with damaged muscles.

In order to provide a more complete picture, regarding the effects and appropriateness of exercise continuation prior recovery, we have designed an integrated study investigating several physiological aspects including mechanical, morphological, and cellular-molecular.

II. OBJECTIVES

The present study aimed to examine the effects of exercise continuation, prior full recovery, on several physiological aspects including mechanical, morphological, and cellular-molecular. This was done in an attempt to examine the appropriateness of exercise continuation with affected and/or damaged muscles.

In other words, we have all experienced at some time the feeling of a sore muscle after an unusual or unaccustomed activity. The question is, whether is proper to continue that particular activity/exercise before skeletal muscle recovery.

The particular objectives of the current study were to determine, in a group of middle-aged untrained individuals, the effects of six repeated-bouts of eccentric exercise on:

- The mRNA expression of myogenic (MyoD, Myogenin, Myf5, and Myostatin) and cell cycle (P21, Ki-67) regulatory genes.
- The indirect (CK and LDH activity, MAT, and DOMS) and direct (myofiber and sarcolemma damage) markers of skeletal muscle damage.

III. METHODS

III. 1. Selection of Subjects

Fifteen healthy, untrained males [age 27.0 years (SD 10.1), weight 72.6 kg (SD 7.9), height 176cm (SD 6.8)] were recruited for this study and assigned to an experimental group (n=9) or a control group (n=6).

According to the selection criteria, none of the subjects had a history of knee injury and, at the time of the study, all were free of orthopaedic abnormalities, completely free of muscle soreness and had not been used any anti-inflammatory and/or other medication . All subjects received verbal and written information prior to their participation concerning all aspects of the study and all possible complications in relation to the muscle biopsies. A written consent form was obtained from all subjects before participating. The study was performed in accordance with the Declaration of Helsinki

(revised in 2000) and was approved by the Semmelweis University Research Ethics Committee.

III. 2. Experimental design

Pre-exercise muscle biopsies and blood samples were taken from all subjects one week before starting the exercise protocol. At day 1, subjects had their first exercise bout that was then performed for six consecutive days. At day 3 after completing the third-day exercise, blood and biopsy samples were collected again. The final biopsies and blood samples were taken 24h after completing the 6^{th}-day of exercise at day 7. The same timeline protocol was followed by the control subjects, with the exception of the resistance exercise.

III. 3. Resistance Exercise

The exercised group subjects attended the laboratory on six consecutive days. In each session, subjects had to complete six sets of 15 single-leg maximum voluntary contractions (MVC), using their non-dominant knee extensors. These sessions lasted approximately 20 minutes, including rest intervals (1min between sets). Subjects exercised while lying in the prone position using a custom-built computer-controlled isokinetic dynamometer (Multicont-II, Mediagnost, Budapest and Mechatronic Kft, Szeged, Hungary). Each eccentric contraction had 120˚ functional range of motion and 120˚/s angular velocity that were set electronically. The anatomical zero (full extension) of the knee equaled 130˚ for the dynamometer (start position), while 10˚ for the dynamometer equaled 120˚ of the knee joint ankle (end position). After each eccentric contraction, the lever arm was returned to the starting point with 60˚/s angular velocity (thus, allowing two seconds rest between each contraction). Throughout the exercise subjects were verbally encouraged to resist the rotating lever arm with maximum effort.
Muscle biopsies were taken in the fasted state after exercise. However, at days when only the exercise protocol was performed, there was no dietary control.

III. 4. Relative mRNA expression

Firstly, RNA was isolated from each sample according to the manufacture's protocol using Tri Reagent (T9424 Sigma Aldrich). Thereafter, two micrograms of RNA from each sample were reverse transcribed in a total volume of 40 µl. The cDNA samples were stored at -20C° for subsequent RT-PCR analysis. The ABI Prism 7000 Sequence Detection System (Applied Biosystems) was used to quantify mRNA expression for each gene. All reactions were set up in duplicate and repeated once.

The relative expression of different mRNAs was determined by relative quantification $\Delta CT = C_T\text{target} - C_T\beta\text{-actin}$, where β-actin represents the reference housekeeping gene.

Pairs of primers and TaqMan probes were designed by Applied Biosystems (inventoried TaqMan® gene expression assays). All assays were purchased from Applied Biosystems and assay IDs are given in parenthesis below:
(MyoD1 (Hs00159528_m1); Myogenin (Hs00231167_m1); Myf5 (Hs00271574_m1); GDF8 (Hs00193363_m1); p21 (Hs00355782_m1); Ki67 (Hs00267195_m1); β-actin (Hs00242273_m1)).

III. 5. Histology and Immunohistochemistry

All samples were fixed in 7% buffered formalin and processed by standard techniques to paraffin wax. For tissue processing, enclosed automatic processor (PathCenter, Shandon) was used according to routine histopathological processing. From paraffin embedded blocks 4 µm sections were cut and laid on silanized (APES, 3-aminopropyltriethoxysilane) slides. In addition to standard haematoxylin- and eosin-stained sections, immunohistochemical staining was performed using fixed, paraffin-embedded tissue sections and antibodies specific for fibronectin (polyclonal, rabbit anti-human, DakoCytomation, A 0245) and desmin (monoclonal, mouse anti-human, clone D33, DakoCytomation, M 0760) antigens. Sections for all biopsies were observed using a Nikon Eclipse, E600, microscope and the chosen sections were photographed with spot camera (Pixera Penguin 600CL Model). All sections were seen blind to the experimental treatment (exercise or control) by three different parties, one of which was independent to the study.

III. 6. Statistical analysis

Mean and SD were calculated for each variable. Normality tests, Shapiro-Wilk's W test, were performed for all dependant variables. All variables, with the exception of MAT, were analyzed by Friedman ANOVA (a non parametric test alternative to repeated measures of ANOVA), since the variables of interest were not normally distributed, followed by the Wilcoxon Matched Pairs Test as a post hoc test, when appropriate. MAT was analyzed with repeated measures of ANOVA, following by a Scheffe test. In all dependent variables time was used as the repeated measures factor with three levels (pre/ex, day 3 and day 7) with the exception of MAT and DOMS were the repeated measures factor, time, had 6 and 8 levels respectively. Dependent variables were: mRNA gene expression of MyoD, Myogenin, Myf5, P21, Ki-67 and Myostatin; CK and LDH activity; mean MAT; DOMS; muscle fibers morphology; muscle fibers fibronectin and desmin staining. Statistical significance was accepted at $P < 0.05$ for and all tests were performed using STATISTICA 7 software package.

IV. RESULTS

IV. 1. Indirect Markers of Muscle Damage

The indirect markers of muscle damage investigated by the present study included: Maximum Average Torque (MAT), serum creatine kinase (CK) and lactate dehydrogenase (LDH) activities, and Delayed Onset Muscle Soreness (DOMS).

MAT was significantly decreased ($P<0.006$) between day 2 and day 5 compared to the first training session. At day 6, MAT recovered significantly from day 3 ($P=0.007$) and was not significantly different from the first training value.

Serum CK and LDH activities were significantly increased throughout the study, although CK was significantly greater at day 7 ($P=0.03$) compared to the day 3 levels. DOMS peaked at day 3 and then gradually decreased such that at day 5 was significantly lower from day 3.

IV. 2. Direct Markers of Muscle Damage

Muscle Histology: All muscle biopsies taken at pre-exercise, at day 3 and at day 7 after three and six consecutive bout of eccentric exercise respectively, showed normal fiber appearance with well-ordered fascicles and tightly packed fibers. In both groups, experimental and control, there was no evidence of gross myofiber lesions. All fibers seen in cross-sections had polygonal appearance with no observable evidence of enlarged and/or rounded fibers. In some cases rounded fibers were observed at the periphery of the muscle biopsy. However, at no instance rounded fibers were observed in the centre of the biopsy. Due to the fact that such fibers were observed in both groups and at all times, the presence of those fibers can only be attributed to the biopsy procedure and not to the exercise. In addition, at no instance those or others fibers were accompanied with mononuclear cells infiltration. There was no evidence of myofiber degeneration and/or necrosis. Only one subject showed evidence of gross myofiber disturbances with infiltration of mononuclear cells, myofiber degeneration and necrosis, all of which commenced at day 7. This subject was the one that displayed extremely high CK activity.

Fibronectin: Plasma fibronectin at no instance was observed within the muscle fibers with the exception of the subject that showed gross myofiber degeneration and necrosis. In this subject, at day 7, the majority of muscle fiber seen in cross and longitudinal sections contained staining of plasma fibronectin.

Despite the increased activities of CK and LDH (described above as indirect markers of muscle damage) at all measurement times, fibronectin staining was never observed within the muscle fibers in eight of nine subjects indicating that there was no sarcolemma disruption. Fibronectin under normal circumstances is excluded from the muscle fibers and has been shown to be an excellent marker for sarcolemma damage. The fact that fibronectin was never observed within muscle fiber suggests that sarcolemma was neither disrupted and neither became permeable for fibronectin. Therefore it appears that the present EE protocol did not induce gross sarcolemma or myofiber damage.

IV. 3. Myogenic regulatory factors (MRFs)

The mRNA expression for MyoD decreased significantly by 45% ($P=0.03$) at day 3 after three bouts of EE, while myogenin mRNA increased significantly by 1.9 fold ($P=0.02$),

compared to the pre/exercise values. The mRNA expression for Myf5 was not significantly changed at any time. The mRNA expression for all MRFs had returned to the pre/ex levels by day 7. Previous studies have shown that resistance exercise induces the expression of MRFs and evidence also suggests that repeated bouts of resistance exercise stimulate greater responses. In the present study, even though, we have utilized a training protocol that was repeated for six consecutive days, the mRNA expression of MRFs was minimal throughout the experimental period.

IV. 4. Myostatin

The mRNA expression for myostatin, a negative regulator of skeletal muscle growth and regeneration, decreased significantly by 74% (P=0.007) at day 3 and by 72% (P=0.01) at day 7. In the present study, we also observed a dramatic Myostatin mRNA down-regulation (~73%) at all measurement times.

Myostatin acts mainly by inhibiting activation, proliferation and differentiation of satellite cells. In light of the latter evidence, we reasoned that myostatin down-regulation would most probably complement increase activity of SCs. SCs, once activated start to express MRFs that are crucial in establishing the myogenic lineage (MyoD, Myf5) and terminal differentiation (myogenin, Myf6) of myoblasts. Consequently, we hypothesized that suppression of myostatin would be an additional reason to expect increased expression of MRFs. Even though the expression of myostatin was dramatically decreased we were unable to support our hypothesis because the expression of MRFs, with the exception of myogenin, was attenuated at all measurement times

IV. 5. Markers of proliferation and differentiation

The mRNA expression for Ki-67 gene, a marker of active cell proliferation, increased significantly by day 3 (2.4 fold, P=0.01) and by day 7 had significantly increased further (12.1 fold, P=0.007). On the other hand the mRNA expression for p21 and myogenin, markers of cell differentiation, increased significantly only at day 3 by 4.1(P=0.007) and 1.9 fold, respectively. At day 7, after six consecutive days of EE, both had returned to the pre/ex levels.

V. CONCLUSIONS

In the current study our aim, as noted, was to examine the effects and subsequently determine the appropriateness of exercise continuation with affected and/or "damaged muscles". Although we observed no gross myofiber and sarcolemma damage in 8 of 9 subjects we cannot ascertain the appropriateness of exercise continuation with affected muscles prior full recovery. This is due to the impaired expression patterns of MRFs that, with the exception of myogenin that showed a moderate non sustained increase, MyoD and MYf5 response was minimal. Under these conditions exercise continuation may be associated with impaired muscle growth and/or regeneration since MRFs are crucial in establishing the myogenic lineage and terminal differentiation of myoblasts; and also regulate the expression of several muscle specific genes like myosin, desmin and troponin.

The results of the present should be viewed with caution since variables like rest interval between exercise bouts; gender; age; activity level; etc. if taken into account may affect differently the applicability of the present findings. More over it should be noted that any conclusion made as a result of the findings of the present study, particularly concerning the myogenic response, is based on mRNA alterations. Whether protein alterations have indeed occurred remains to be elucidated.

Therefore, issues like the aforementioned should be examined by future studies to provide more information regarding the continuation of exercise with affected muscles.

Based on the presented findings and within the limitation of this study, we conclude that:

- Repeated bouts of eccentric exercise, for six consecutive days, dramatically decreased Myostatin mRNA expression but impaired the expression patterns of MRFs such that, with the exception of myogenin that showed a moderate non sustained increase, MyoD and MYf5 response was minimal.
- Repeated bouts of eccentric exercise, for six consecutive days, do not cause gross sarcolemma damage in the mid-belly of Vastus Lateralis, in humans.

OWN PUBLICATION LIST

A) Articles related to the PhD thesis:

Costa A., Dalloul H., Hegyesi H., Apor P., Csende Z., Racz L., Vaczi M., Tihanyi J. 2007. Impact of repeated bout of eccentric exercise on myogenic gene expression. *Eur J Appl Physiol*. 101(4):427-436

Costa A., Orosz Z., Apor P., Csaba N., Siamilis S., Csende Z., Racz L., Tihanyi J. 2008. Impact of Repeated Bouts of Eccentric Exercise on Sarcolemma Disruption in Human Skeletal Muscle. *Acta Physiol Hung.* [Epub ahead of print]

B) Articles not related to the PhD thesis:

Siamilis S., Jakus J., Nyakas C., **Costa A.**, Mihalik B., Falus A., Radak Z.(2008). The effect of exercise and oxidant-antioxidant intervention on the levels of neurotrophins and free radicals in spinal cord of rats. *Spinal Cord. [Epub ahead of print]*

Apor P., Tihanyi J., **Costa A.** (2007).Improvement of muscle mass and force by certain hormones and endogen factors, contributing in muscle development. *Orv Hetil. 148(10):451-6*

Tihanyi J., **Costa A**., Váczi M., Sáfár S., Rácz L. (2008). Active torque enhancement during voluntary eccentric contraction. *MSTT. 34:15-25*

C) Conference Proceedings (abstracts):

Costa A., Hicham D., Zsolt C., Hegyesi H., Tihanyi J. (2006). Impact of chronic eccentric exercise on MRFs, myostatin and MYH3 mRNA expression in human skeletal muscle. Proceedings of 11th annual Congress of the European college of Sport Science, 137-138.

Costa A., Hicham D., Zsolt C., Tihanyi J. (2005). Indirect markers of skeletal muscle damage by 6-days eccentric exercise. Proceedings of the 5th annual Congress of Sport Science.

Racz L., Vaczi M., Costa A., Safar S., Tihanyi J. (2006). Effect of stretching velocity on active and passive force enhancement. Proceedings of the 11th annual Congress of the European college of Sport Science, pp 373.

Tihanyi J., Racz L., Trzaskoma L., Costa A. (2006). Influence of passive force enhancement on active force enhancement. Proceedings of the 11th annual Congress of the European college of Sport Science, pp 459.