Functional and Histopathological Changes in Muscle after 6-weeks Repetitive Strain Injury: A 10-week Follow Up of Aged Rats

Taher Afsharnezhad1*, Maryam Nourshahi2, Siavash Parvardeh3

1,2 Department of Exercise Physiology, School of Physical Education and Sport Sciences, Shahid Beheshti University, Iran
3 Department of Pharmacology, School of Medicine, Shahid Beheshti University of Medical Sciences, Iran

ABSTRACT: Repetitive eccentric contractions are associated with repetitive strain injury (RSI) of muscle and tendon and were accompanied by an increase in extracellular matrix (ECM), atrophy, and reduce force. However, a research gap exists regarding the effect of aging on injury susceptibility and recovery to repetitive strain exposures. In this paper, we examined the response of gastrocnemius of aged rats to 6 weeks chronic strain injury followed 10 weeks without specific rehabilitation. 16 elderly male rats divided to two groups: control (n=8) and RSI (n=8). RSI group underwent 6 weeks (5 days/week) of fast velocity submaximal eccentric contractions. After 4 and 10 weeks’ post-injury non-active rest, isometric force, muscle wet mass, and histopathological changes of gastrocnemius muscle in RSI-model and control groups were measured. After 4 weeks’ post-injury raw and relative (percent to body weight) measures of isometric force and wet muscle mass of gastrocnemius in control group are significantly greater than RSI group. Force deficit was reduction Masson Trichrome and Hematoxylin & eosin stains also showed histopathologic changes were present only in RSI group that included increase in fibrosis and non-contractile area, and decrease of myofiber area. After 10 weeks of injury protocol, decrease in IF of gastrocnemius (8% and 6% for raw and relative measures respectively) were remained in RSI-Re group, but muscle wet mass was recovered. Also, myofiber area and non-contractile area were not fully recovered after 10-week rest in RSI-Re group (+2.77% and -3.6% respectively). Six weeks repeated bouts of moderate eccentric contractions caused in the rat gastrocnemius muscle decreases in muscular size and strength and myofiber area, whereas the non-contractile area and fibrosis was markedly increased. These results suggest that in aged rat force deficit and histopathological changes of gastrocnemius muscle after chronic strain injury were reminded after 10 weeks’ rest. These observations are consistent with the hypothesis that structural damage (e.g. fibrosis) was not recovered after prolong rest.

KEY WORDS Repetitive strain injury, Eccentric contractions, Aging, Histopathological changes

INTRODUCTION

Repetitive strain injury (RSI) is one of the musculoskeletal injuries occur in nerve, muscle, tendon and bone due to low-intensity, high-speed, repetitive eccentric contractions (REC) [1]. RSI, also called overuse injuries, is prevalent among athletes and industrial workers. Common symptoms in these patients include weakness, pain, tenderness, throbbing and tingling sensations, a feeling of stiffness and fatigue in muscle that develop and become constant gradually[2].

The four primary risk factors of RSI are the force, repetition (overuse), velocity, and range of motion. Besides these, there are several other risk factors such as aging, poor posture[3], stress[4], obesity[5], hypertension, heart disease,
loose-jointed, insomnia and a sedentary lifestyle can also be effective on RSI[6, 7].

REC lead initially to an inflammatory response[8]. While the result of inflammation is to repair injured tissues, continuing repetitive task develop a vicious cycle of injury and cause chronic inflammation[9]. The final result is often loss of motor function. The actual underlying mechanism that results in strength loss is not understood. However, studies showed evidence of degenerative changes in muscle, peripheral nerve, and central nervous system[2]. Adverse changes in muscle tissue include atrophy, fibrosis, fat infiltration and perhaps even tissue breakdown. RSI results in expansion of extracellular matrix and collagen deposition around the myofibers and fiber necrosis[10]. The ECM provides support to contractile tissue and help to activate and migrate satellite cell[11]. After RSI, synthesis of type I and III collagen within muscle were increased[12]. Thus, chronic inflammation can trigger an excessive accumulation of ECM components, which tend to the formation of a fibrotic tissue[13].

Aging always is a key factor in the overuse injury. In the elderly people, the regenerative abilities of skeletal muscles deteriorate[14]. In chronic injury condition, the healing process is inefficient, because of reduced the number of muscle stem cells and increasing expression the TGF-β superfamily in aging[15, 16]. Satellite cells are the primary source of muscle regeneration. Reducing activated satellite cells with aging lead to replacing muscle by adipose and fibrotic tissue[15]. The expression of TGF-β1 and Myostatin lead to increase in P-Smad3. P-Smad3 and activated Notch in satellite cells compete on controlling transcription of Cyclin-dependent kinase (CDK)[17]. During aging, P-Smad3 wins and the CDK inhibitors were increased that result in decrease the proliferation of satellite cells[18]. Thus, the number of satellite cells decrease, and this is the main cause for decline anabolic response in the elderly. Furthermore, pro-inflammatory cytokines accelerate degradation and inhibit protein synthesis, leading to slower repair and reduced adaptation of older skeletal muscle to overuse injury[15]. Finally, these changes enhance fibrogenesis compared to the myogenesis process. These pathological changes associated with strength loss, chance of re-injury, and increases healing time[19].

There is little information about pathophysiology underlying the prolonged degenerative changes of repetitive strain injury in aging. This changes do not heal completely after several months' rest. Thus, RSI may need more aggressive intervention including surgery and can persist for years. In this study, we examined the response of gastrocnemius of aged rats to six weeks' chronic strain injury followed 10 weeks' rest without specific rehabilitation.

**METHODS**

In an experimental design, 32 elderly male Wistar rats (410.13±25.68 g, 20-22 months old) were divided into four groups randomly (CTL-1, RSI-1, CTL-2 and, RSI-2). Two RSI groups underwent 6 weeks of fast velocity submaximal eccentric contractions by electrical stimulation while the two control (CTL) groups were inactive. After 14 days' post injury, one of the RSI (RSI-1) and one of the control (CTL-1) groups were sacrificed for initial assessment of RSI-induced adaptations. Both RSI-2 and CTL-2 groups group were inactive without any modalities for 10 weeks for Long-term assessment of RSI effects. The care and use of the Rats was followed the guidelines of the Shahid Beheshti University of Medical Science.

**RSI Protocol**

For each RSI session, rats were anesthetized and stimulated using a manual training program. Electrodes for electrical field stimulation were
inserted subcutaneously and positioned along the surface of the gastrocnemius muscles of the right hind limb. Electrical stimulation with 200 ms pulses at 70Hz and 40V was used. Repeated eccentric contractions of activated gastrocnemius muscles were performed by ankle rotations with an angular velocity of 225°s⁻¹ from an ankle position of 140° to 40° and returned. Each session consisted of 5 bouts of 10 eccentric-concentric cycles with a rest period of 30 s.

**Maximal Isometric Force**

Isometric force (IF) measurements were taken under Ketamine/xylazine-induced anesthesia before sacrificing the Animals. Using an ankle position of 90°, a contraction duration of 600 ms, a stimulation frequency of 120 Hz, and 70V, Force of the plantar flexor muscle was measured as a reaction force under the sole of the rat’s foot.

**Wet Muscle Mass**

At the two specific times, groups of animals were weighed, exsanguinated under anesthesia (Ketamine/xylazine at 12 mg/100 g body weight), and sacrificed after cardiac perfusion with saline, followed by a 10% formalin flush. The gastrocnemius muscles were dissected from both hind limbs, cleaned and individually weighed. The weight of each muscle was normalized to the animals’ body weight.

**Histological Analysis**

Animals were fixed by transcardial perfusion with buffered formaldehyde. Gastrocnemius muscles were removed and were fixed in 10% buffered formalin. After 1 day of fixation, Specimens were sectioned in the transverse plan with a surgical blade through the center of the muscle and subjected for tissue preparation. These samples were paraffinized on special cassettes and sectioned at 5 µm by microtome. Then the sections collected on glass slides.

Quantitative histopathological analysis was done by Masson Trichrome (MT) and Hematoxylin & eosin (H&E) staining, and myofiber area (MA) and non-contractile area were determined by ImageJ software.

**Statistical Analysis**

Values were reported as mean ± SD. Statistical analysis was performed using one-way analyses of variance (ANOVA). When a significant F-ratio was found, post hoc testing was carried out with Tukey's HSD tests. Differences were accepted as significant at P<0.05. All statistical testing was performed using IBM SPSS statistics (version 23, SPSS Inc., Chicago, IL).

**RESULTS**

Raw and normalized (to body mass) isometric force decrease significantly in RSI-1 group (17% and 9% respectively). This reduction in IF was not fully recovered after 10 weeks' rest (7% and 7.5% respectively).

Also after two weeks, wet muscle mass of gastrocnemius in CTL-1 was significantly greater than RSI-1 (23% and 17% for raw and normalized measures respectively). After 10 weeks, the weight of the gastrocnemius muscle of the right hind-limbs were similar in both RSI-2 and CTL-2 rats.

Masson Trichrome and H&E stains also showed histopathologic changes were present in RSI-1 group that included increase in fibrosis and non-contractile area (6.16%), and decrease of myofiber area (10.61%). After 10 weeks of injury protocol, myofiber area and non-contractile area were not fully recovered after 10-week rest in RSI-2 group (-5.35% and 3.77% respectively).
FUNCTIONAL AND HISTOPATHOLOGICAL CHANGES AFTER REPETITIVE STRAIN INJURY

Figure 1. Maximal Isometric Force of Plantar Flexor muscle during recovery after 6 weeks of chronic strain.

Figure 2. Wet Muscle Mass of gastrocnemius muscle during recovery after 6 weeks of chronic strain.

Figure 3. Myofiber and non-contractile area of gastrocnemius muscle during recovery after 6 weeks of chronic strain.
DISCUSSION

The results of our study demonstrate a relatively high strength loss, reduced gastrocnemius muscle myofibrils area and, increased extracellular matrix after 6 weeks' repetitive eccentric contraction in elderly rats. These results were seen after two weeks of non-active post-injury rest and were not recovered after 10-weeks. These findings are consistent with other animal studies[20, 21] and clinical trials on human subjects[22]. For example, Williams and Stauber (2009) reported force reduction followed by repetitive eccentric contractions[21]. In the another study, they reported histopathological change prevention by increasing the rest time between contractions). In this study, we used low rest intervals (30s) and a long time protocol of RSI[20].

The results of this study also showed that some degenerative changes such as atrophy was seen after 2 weeks, and was recovered after a 10 weeks of rest. It is believed that excessive accumulation of calcium caused by repetitive eccentric contraction may result in histopathological changes in muscle tissue[23]. Ca$^{2+}$ overload may result in activation of Ca$^{2+}$-activated neutral proteases (calpains) that is closely associated with the I and the Z band regions and calpain-mediated degradation is thought to contribute to the changes in muscle structure[24, 25]. Specifically, titin and α-actinin, two sarcomeric proteins, are readily proteolyzed by calpains[26].

The results of this study also showed that after 10 weeks, increasing in ECM and reducing of contractile area were not fully recovered. This finding confirms the persistence of the histopathological change of muscle after chronic strain in elderly rats. Collagen take part in muscle recovery by forming a sheath around myoblasts during myotube formation[27]. When RSI produce excessive collagen in muscle, a mechanical barrier to regenerating muscle fibers may be created[27]. After 6 weeks of repetitive strain injury, non-contractile area increased almost 6%. Although non-contractile area decreased almost 2.4% with recovery time. The myofiber area percent reached a 65.8% at 2 weeks of recovery (10.6% decreased) and remained less than CTL even at 10 weeks (5.35%). Some researcher reported loss of strength and muscle mass with a reduction in myofibrillar area and an increase in extracellular matrix after repetitive strain injury[28-30]. In contrast, degenerative changes were not being reported in some studies after RSI[30].

Skeletal muscle repair following injury is a balance between fibrosis and regeneration[27]. Factors affecting this balance include of inflammation, the growth factors and cytokines present in site of injury, and the interaction between infiltrating inflammatory cells and native myogenic cells[31]. TGF-β superfamily increase in muscles under conditions of chronic strain and overload injury, and has been associated to the muscle fibrosis[32]. Smith et al. reported increasing TGF-β1 in skeletal muscles 48 h after repetitive strain injury[33]. Both TGF-β1 and myostatin have been shown to regulate maladaptive type muscle repair and myogenesis[34]. Muscle fibrosis is a final outcome of activating TGF-β family and Smad3 pathway[35]. After repetitive strain injury, Myostatin, a negative regulator of myogenic process, was increased that replace activating of myogenic with Fibrogenic cells[34].

In conclusion, these findings show that the most of outcomes of RSI were reminded after long time of rest without any modalities. Further study is needed to develop interventions for muscle fibrosis due to chronic muscle injury.
REFERENCES


2. Hadrévi, J., Applying proteomics and metabolomics for studying human skeletal muscle with a focus on chronic trapezius myalgia, in Faculty of Medicine, Department of Integrative Medical Biology (IMB). 2012, Umeå University: Umeå, Sweden. p. 60.


