Effects of eccentric exercise on different slopes

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Introduction

Eccentric contraction occurs when the external force acting on the muscle is greater than the force produced by contraction or can be defined as an active lengthening of muscle while the muscle tone increases¹. It allows absorption of mechanical energy, which is an essential feature in locomotion when the body needs to decelerate like walking downhill or descending stairs². Eccentric contraction elicits a high level of muscular force compared to isometric or concentric contraction³ and yet has relatively low metabolic, hemodynamic, and ventilatory demand⁴. Therefore, eccentric contraction dominant exercise protocols (aka. eccentric exercise or eccentric training) are widely used to overload the muscular system with a little energy cost. Eccentric exercise not only has an improving effect on the performance and strength of elite athletes⁵ but also has a preventive effect against injuries⁶. Besides, it is more useful for the recovery of orthopedic rehabilitation of tendinopathy and anterior cruciate ligament injuries compared to concentric exercise⁷-⁸ and it is suitable for recovery of muscle mass and strength in aging and critically ill population⁹,¹⁰.

On the other hand, it is well known that eccentric exercise induces muscle damage and delayed-onset muscle soreness (DOMS) in sedentary individuals. DOMS is the pain and stiffness felt in muscles several hours to days after an exercise session, especially after unaccustomed or strenuous exercise. Progressive necrosis of the contractile elements¹¹, the release of prostaglandins¹² and enhanced responsiveness of nociceptive endings are the leading causes of DOMS¹³. Muscle damage and DOMS together impair the voluntary force generation ability of the muscle¹⁴. 

Abstract

Objectives: Eccentric contraction occurs when the muscle lengthens under tension. Damage-induced responses seen in the muscle after eccentric exercise usually experienced by sedentary individuals. This study aims to investigate muscle damage on different slopes. Methods: 32 male Wistar albino rats randomly divided into four groups: sedentary, horizontal running, and eccentric exercise (-8°, -16°) groups. Animals ran for 90 min with the speed of 25 m/s for five days. After 48h from the last exercise, rats were sacrificed, and plasma creatine kinase (CK), heat shock protein 70 (HSP70) levels were examined. Plasma and soleus total oxidant/antioxidant status (TOS-TAS) and histological changes of soleus muscle assessed. Results: CK and HSP70 significantly increased in 16° EE group. TOS increased at 16° EE and 8° EE, but oxidative stress index (OSI) was only high at 8° EE group. Mononuclear cell infiltration and the angiogenesis increased in soleus after eccentric exercise, and there was a correlation with slope. Sarcomere breaks were detected in 16° EE group also in a correlation with slope. Conclusions: Consequently, sedentary individuals are vulnerable to injuries induced by eccentric contraction. Therefore, our study provides information for reconsidering rehabilitation and training programs.

Keywords: Creatine Kinase, Eccentric Exercise, HSP70, Muscular Damage, Small Animal Treadmill

The authors have no conflict of interest

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The magnitude of muscle damage is determined by exercise intensity, genetic variations, active muscle group, or type of exercise (voluntary or electrical stimulation). Creatine kinase (CK) is the most common and widely used biochemical quantitative indicator of muscular damage. It was observed that the elevation of the plasma CK, began immediately and reached its peak after 48 hours from the last eccentric exercise session.

Physical activity increases the quantity and activity of stress proteins, including the Heat Shock Proteins (HSPs). HSPs are known to be expressed in various tissues (lung, kidney, heart, skeletal muscle, etc.) and takes part in the endogenous defense system, aids the antioxidant system and assists a wide range of protein folding processes. One of the most widely studied heat shock protein is HSP70. Paulsen et al. reported that plasma HSP70 level was increased 4 hours after training and stayed elevated for days after a single bout of exercise. Lollo et al. showed that eccentric contraction is more potent in inducing the HSP70 response than concentric exercise or horizontal running. However, it is still unknown if there is a relationship between the degree of negative slope and the plasma level of HSP70.

Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) and the buffering capacity of antioxidant defense mechanisms thus resulting in a local imbalance between ROS production and destruction. Although oxidative stress is not a direct indicator of muscle damage still, it is closely related to muscular injury and a valuable tool for evaluating the effect of exercise both on tissue and organism. Some studies are reporting that DOMS is closely related with redox status and application of antioxidants ameliorate the DOMS and there are few studies about eccentric exercise and oxidative stress, and they conclude that eccentric exercise increases oxidative stress and this effect is more prominent in sedentary individuals.

In this study, we hypothesize that the muscle damage caused by eccentric exercise will increase in parallel with the degree of negative slope. For demonstrating muscular damage, we aim to observe the possible alterations in redox status, CK, HSP70 molecules, and histological appearance of tissue, all of which would reflect possible muscular damage.

Materials and methods

Animals and ethical approach

Male Wistar albino (Rattus norvegicus) rats (n=32) aged 12 weeks were used in this study. Animals were kept in a 12-hour light/dark cycle at constant temperature (20-22°C) and humidity (45-50%), had access to ad libitum chow and tap water. All animals were handled in accordance to the Guiding Principles in the Care and Use of Animals of the American Physiological Society. All procedures were approved by the Committee on the Local Ethics of Animal Experiments of Ankara University Faculty of Medicine (No: 2013-15-119).

Animals were randomly assigned into four groups which are sedentary (S), horizontal running (0° HR), -8° eccentric exercise (8° EE), and -16° eccentric exercise (16° EE).

Treadmill exercise

Animals have been habituated to treadmill running in a week. After a 3-day rest, the training protocol (adapted from Armstrong et al.) was initiated (Table 1). Briefly, the speed is gradually increased in first 15 minutes and the animals exercised on the treadmill for another 75 minutes with 20-25 m/min at 0° slope, down a -8° slope (8° EE) or down a -16° slope (16° EE). This protocol is repeated daily for five days. During this time sedentary animals were kept in their cages.

Biochemical parameters

Forty-eight hours after the last exercise session animals were anesthetized with sodium thiopental (50 mg/kg; i.p.) and sacrificed by opening the chest and removing the heart.
The blood filled into the chest cavity was collected rapidly and centrifuged at 3000 g for 15 minutes at +4°C to obtain plasma. Plasma samples were kept into -80°C for further analysis. The left pair of soleus muscle was removed and snap frozen by liquid nitrogen and kept at -80°C for further analysis. The right pair was also removed and stored in 10% buffered formalin solution for histological evaluation.

**Markers of muscular damage and protection**

Plasma Creatinine Kinase (CK) and Heat-Shock Protein-70 (HSP-70) concentration were determined with ELISA according to the manufacturer’s instructions (Cusabio Technology LLC, Texas, USA).

**Redox status measurements**

Plasma total oxidant (TOS) and antioxidant status (TAS) were measured by special colorimetric assay kits according to manufacturer’s instructions (Rel Assay Diagnostics, Gaziantep, Turkey). Oxidative stress index (OSI) was calculated simply according to the formula: OSI=TOS/TAS

Soleus muscle samples were homogenized before assays according to a specific homogenization protocol. Tissue samples were weighed and pretreated with 9 ml of homogenization buffer (0.1 M KCl) per 1 g tissue. Tissues were manually homogenized on ice with a Teflon homogenizer (Glas_Col K5424). Homogenates were centrifuged for 15 min at 3000g, and the supernatants were used for TOS and TAS analysis.

**Histopathological examinations**

a. **Light microscopy**

Soleus muscles were fixed in 10% buffered formalin. Samples were dehydrated in a graded ethanol series, cleared in xylene, and embedded in paraffin. Sections were cut in 5-μm thickness using a microtome (Leica RM 2125RT) and stained with hematoxylin and eosin (H&E). Slides were examined and photographed using an Axio Scope-A1 (Carl Zeiss, Oberkochen, Germany) microscope.

H-E stained sections were scored from 0 (none) to 4 (severe) (score 0: none, score 1: mild, score 2: positive, score 3: strong positive, score 4: severe positive) to determine the degree of vascularity and inflammation of the tissues. Muscle fiber breaks and myotube formation were also observed.

b. **Transmission electron microscopy**

Samples were fixed with phosphate buffered (pH 7.4) 2.5% glutaraldehyde and 2% paraformaldehyde mixture solution for 2-4 h at room temperature. Then they were washed with a phosphate-buffered saline solution (PBS, pH 7.4) and fixed with 1% osmium tetroxide for 2 hours as the secondary fixative. Subsequently, they were embedded in Araldite 6005 and cut with a Leica Ultracut R ultramicrotome (Leica, Solms, Germany). One micrometer semi-thin sections were stained by toluidine blue-Azur II to select the region of interest for the following procedures. 60-70 nm thin sections of selected regions were stained with uranyl acetate and lead citrate. These sections were examined to see Z lines and photographed using a LEO 906 E TEM (80 kV, Oberkochen, Germany) microscope.

**Statistical analysis**

Graph Pad Prism (Prism 5 for Windows, GraphPad Software, USA) was used for the analysis. Univariate analysis of variance (ANOVA) was used to analyze all of the parameters Tukey test used as post hoc. p<0.05 was considered as statistically significant. Histopathological parameters were evaluated blindly and analyzed by Kruskal Wallis and the Mann-Whitney U tests. Data were presented as mean ± standard deviation (X±SE).

**Results**

1. **Markers of muscular damage and protection**

Plasma CK level, which is the cardinal indicator of muscular damage was significantly increased in 16° EE group compared to all other groups (p<0.001). Although a slight increment was observed in other running groups (0° HR and 8° EE), it was not significant (Figure 1A).

A similar pattern has emerged in HSP-70 results. 16° EE group had the highest plasma HSP-70 level, and it was significantly different compared to sedentary animals (p<0.05).

Moreover, again, there was no difference between the other three groups (Figure 1B).

2. **Redox status measurements**

In plasma samples, TOS was significantly high both in 8° EE and 16° EE groups compared to the sedentary group (p<0.05). TOS increment mentioned above was counterbalanced significantly by an antioxidant capacity increase in 16° EE (p<0.05) but not in 8° EE group. This contrast was also reflected in OSI result, which is the primary indicator of oxidative stress. The OSI index was significantly higher in 8° EE (p<0.05) but not in other exercise groups, compared to sedentary animals (Figure 2A, 2B, and 2C).

In soleus muscle samples, TOS was significantly high only in 16° EE group compared to the sedentary group (p<0.05).

In TAS results, there was a difference between 8° EE and 16° EE groups, and 8° EE has the lowest antioxidant capacity. Like the plasma, in tissue samples, a dramatically high OSI level was observed in 8° EE group, it was significantly high compared to all other three groups (p<0.05).

3. **Histopathological examinations**

Representative light micrographs of all groups were given in Figure 3, and representative TEM micrographs of all groups were given in Figure 5.
Figure 1. Plasma levels of muscular damage and protection markers (A) Creatine Kinase (B) Heat Shock Protein-70 (HSP-70) (*: p<0.05 vs. Sedentary).

Figure 2. Redox status measurements. Plasma (A) Total Oxidant Status (B) Total Antioxidant Status (C) Oxidative Stress Index. Soleus muscle (D) Total Oxidant Status (E) Total Antioxidant Status (F) Oxidative Stress Index (*: p<0.05 vs. Sedentary; ¥: p<0.05 vs. 16° EE).
a. Light microscopy

Histological examinations of soleus muscle micrographs in the sedentary group showed that the nuclei were located peripherally, fibroblasts and capillary structures were observed in the endomysium, and the number of mononuclear cells in skeletal muscle were limited.

Unlike the sedentary rats, in exercise groups, especially in 16° EE, breaks in skeletal muscle fibers became evident, and the frequency of breakage was increased parallel to the slope. There was a prominent myotube formation between some muscle fibers, and the nuclei were located centrally.

There was a significant increase in vascularity and the downhill running groups compared to sedentary animals (p<0.001) which is a well-known effect of exercise, on the other hand, we couldn’t observe the same effect in the horizontal running group. Mononuclear cell infiltration was also increased significantly in the downhill running groups but not in the 0° HR group compared to the control (p<0.001) (Figure 4).

b. Transmission electron microscopy (TEM)

Typical muscular structure and regularly arranged Z lines were observed in sedentary and horizontal running groups
Figure 4. Scores of histological parameters. (A) Mononuclear cell infiltration (B) Vascularization. (0: none, 1: mild, 2: positive, 3: strong positive, 4: severe positive). (*: p<0.05 vs. Sedentary)

Figure 5. Representative TEM micrographs of all groups. Downhill runner groups show irregular arrangements of Z lines. (A) Sedentary (B) 0° HR (C) 8° EE (D) 16° EE. (Arrow: Z lines, m: mitochondrion).
according to TEM micrographs. However, there was an irregular alignment of Z lines in downhill running groups. The irregularity was parallel to the slope dramatically increased, especially in thin sections of the 16° EE samples.

**Discussion**

The main result of the present study demonstrated that eccentric treadmill exercise damages soleus muscle in rats, and this damage increases in parallel with the degree of negative slope. This conclusion was supported by our data, which shows an elevation in plasma CK levels, and oxidative stress and the muscular damage was also evident in histopathological observations.

Plasma CK level, which is the primary indicator of muscular damage was significantly high at 16° EE group. Even though a slight increment was observed in 8° EE and 0° HR groups, it was not as prominent as 16° EE group and could not reach the significance. This finding proves that CK levels are closely related to the intensity of eccentric contraction. Our data is consistent with some studies in the literature, which also shown CK increase at -16° negative slope after 90 minutes from the last exercise session. Additively, Armstrong et al. reported an increase in the plasma CK level with a similar protocol at -16° slope but measured the highest CK level after 36 hours from the last exercise. Magalhaes et al. observed the highest CK level after 48 hours from eccentric exercise with a different exercise protocol and Lollo et al. found a significant increase in the CK level in -7° negative slope at the end of the 6th hour compared to the control and 0° groups. Touchberry et al. used an intermittent running protocol (-16°, 5 minutes running, 2 minutes rest, 18 sets) and the CK peak was prominent after 90 minutes, and it took 48 hours to return to control values. Newham et al. claimed that the degree of muscle extension during eccentric exercise affects the degree of strength loss. His findings pointed out that there was a positive correlation between the slope and loss of strength. We weren't able to measure the loss of strength that was one of the limitations of our study but it can be considered that if the change of CK level is related to the degree of the slope and since it is the primary marker of muscular damage, it also may have related with the loss of strength. Although, Delp et al. pointed out that, exercise-induced CK change is associated with type 2 muscle fiber damage, we were able to demonstrate the similar results with rat soleus muscle of which predominant fiber type is Type I.

It is a well-known fact that the heat shock proteins are one of the most critical biomechanical and molecular stress response elements of the muscle cell. According to our data, HSP70 levels were increased correspondingly with CK levels. Plasma HSP70 concentration was significantly high in 16° EE group compared to the sedentary group, although there were slight increments in 0° HR and 8° EE groups it was not significant. HSP70 concentration usually increases after exercise because HSP70 takes part in protein synthesis and muscular hypertrophy and has been shown to increase muscle weight, and fiber size. However, the magnitude of HSP70 increase was not the same in all running groups; another possible explanation is also needed. Concerning concurrent CK increment, especially in 16° EE group, we hypothesize that HSP70 may also form a compensatory defense mechanism against muscle injury in the muscles which are exposed to high level of mechanic stress. Touchberry et al. observed increased HSP levels in soleus muscle after 2 hours of downhill running exercise, but not after 48 hours, but we were able to observe an increase after 48 hours. Even though there is an alteration in plasma HSP70 level, we have to acknowledge that the tissue level of HSP70 would have given more specific and reliable information about the protective mechanism in the soleus muscle. We are well aware of the fact that HSP70 alterations aren't solely originated from soleus muscle, so we are paying attention to discuss this matter as an effect of eccentric exercise on the whole organism. Unfortunately, we were not able to measure the HSP70 concentration in tissue because of technical impossibilities (samples lost because of freezer malfunction) this is one of the limitations of our study.

It has been reported that eccentric exercise increases ROS. Free radicals are known to be an essential part of the muscular damage process, and they can cause damage by infiltrating lipid membranes, which results in cell necrosis. At the same time, there is an expanding literature that suggests free radicals act as signaling molecules which are necessary for muscle regeneration and adaptation following damage.

According to our study, the TOS value which is an indicator of increased ROS production was significantly high in 16° EE group both in plasma and tissue samples and increased ROS production was counterbalanced with an increase in antioxidant capacity. As a result, OSI of 16° EE group wasn't significantly high compared to other groups. Nevertheless, in 8° EE group has shown a significant ROS increase, which was not counterbalanced because of that OSI of 8° EE group was significantly high compared to other groups both in plasma and tissue. This means the intensity of eccentric exercise in 8° EE group is not sufficient to stimulate an increase in antioxidant capacity. Even though the highest muscle damage is observed in 16° EE group, in the long term this group may be advantageous against 8° EE group because of their stimulated antioxidant system, and it should also be noted that HSP70 levels of 16° EE group were higher compared to 8° EE animals. At this moment, we recommend extended exercise protocols to test this hypothesis.

Eccentric exercise causes neutrophil infiltration, Z line discontinuities, mononuclear cell clusters, T tubular anomalies, sarcomere breaks and design loss. We observed significantly high mononuclear cell infiltration in the 8° EE and 16° EE groups and mononuclear cell clustering around perimysium and endomysium was increased with slope. We also found breaks in the sarcomere lines consistent with the observation of Talbot and Morgan especially in 16° EE group but not in other groups. Z line discontinuities which were observed 8° EE and 16° EE groups were consistent with the eccentric exercise literature. Taken together, we can say there is substantial histological evidence about muscular...
damage and severity of injury increases with the slope.

Angiogenesis is closely related to metabolic demand, which is expected to be low in eccentric contraction. Nevertheless, we observed an increased number of capillaries around the muscle fibers parallel to the slope. Although eccentric exercise demands less systemic $O_2$ local oxygenation of the muscles and metabolic cost may not be correlated, or this may be related to a dysfunction in microcirculation or inflammatory vasodilator response.

Last but not least, we want to emphasize some imperfections of our study. First of all, we have planned a warm-up period to minimize the injuries non-related with the eccentric contraction. Simply, we increased speed and slope gradually before reaching target exercise. Instead of this, we may have chosen a uniform warm-up protocol for all groups to prevent unwanted confounding factors. Secondly, we adapted animals to treadmill running before exercise protocol with a very light adaptation program to prevent any training effect. Even though we don’t think the adaptation program created any training effect, we may have applied the same adaptation program to control animals just to make sure to eliminate the effect of adaptation. Nevertheless, we can consider that our horizontal running group eliminates these effects.

Conclusion

Eccentric exercise widely used as a treatment protocol for orthopedic injuries like tendinopathy or anterior cruciate ligament ruptures or as a training program for elite athletes. Our results show that eccentric exercise causes muscle damage, and the severity of the injury is closely related to the degree of slope. On the other hand, we would like to indicate that our exercise protocol cannot easily be adapted to a clinical setting. Even though we demonstrated muscle damage, our exercise protocol was more intense compared to human exercise programs. Therefore, we think further studies are needed before rushing to a negative conclusion about eccentric exercise. Finally, at least we can say the degree of slope must be chosen carefully in the planning of training and treatment protocols to minimize the negative effect of eccentric contraction. As a future perspective, further work can be useful for evaluating the functional changes in soleus muscle with eccentric exercise.

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Author Contributions

Design of the work: EvG, ADD, EK. Collection of Data: EvG, FA, EmG, PB. Analysis and Interpretation of Data: EvG, FA, EK, PB, DB. Drafting and Revis-ing: EvG, FA, EmG, ADD, DB, EK. Final Approval: All authors.

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