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HEALTH SCIENCES

Protective effect by low-intensity downhill running training against muscle damage and oxidative stress after high-intensity downhill running in rats

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Abstract: This study examined the effects of low-intensity eccentric exercise training performed before high-intensity eccentric exercise on muscle damage markers, oxidative stress and antioxidant defense. Twenty-two rats were divided into 3 groups; control (CON; n = 6), high-intensity eccentric exercise (HE; n = 8) and low-intensity eccentric exercise training plus high-intensity eccentric exercise (LET + HE; n = 8). Rats in the HE group performed HE at once. Rats in the LET + HE group performed LET and then HE protocol was applied. Blood and vastus intermedius muscle samples were taken 24 hours after the last exercise session for analyses of muscle damage, oxidative stress, and antioxidant defense markers. Muscle damage markers were higher in the HE group than the CON (137%-488%) and the LET + HE groups (82%-110%) ($P < 0.05$). Oxidative stress marker was higher in the HE group than the CON (65%) and the LET + HE (50%) groups ($P < 0.05$). Antioxidant defense markers were higher in the LTE + HE group than the HE group (39%-51%) ($P < 0.05$). In conclusion, low-intensity eccentric exercise training performed before high-intensity eccentric exercise conferred a protective effect against muscle damage by reducing oxidative stress and increasing antioxidant defense.

Key words: eccentric exercise, muscle damage, malondialdehyde, antioxidant defense, repeated bout effect.

INTRODUCTION

Eccentric contraction is defined as the force generated muscle activation involving lengthening of a muscle (Duoglas et al. 2017). During the eccentric contractions although fewer motor units are active, greater power are generated (Enoka 1996). As a result, active muscle fibers and non-contractile structures are exposed to higher mechanical stress, which weakens the cell membrane and organelles and disrupts myocyte homeostasis. This phenomenon is called eccentric exercise-induced muscle damage (EIMD) (Clarkson & Hubal 2002).

Reactive oxygen species (ROS) are generated during normal physiological processes and under normal physiological conditions, the organism has sufficient antioxidant defense to deal with the production of ROS (Strobel et al. 2011). However, these products can become toxic when there is a deficiency in naturally occurring antioxidant defenses or when oxidant species are over produced (Valko et al. 2007). It is generally accepted that when exercise is exhausting, it can cause an imbalance between the oxidant and antioxidant state known as oxidative stress (Leeuwenburgh & Heinecke 2001). Eccentric contractions have also been reported to cause an increase in oxidative

stress in blood and muscle tissue (Quindry et al. 2011). Although oxidative stress may mediate cell damage, exercise-induced cell damage can also stimulate oxidative stress, since during the inflammatory response to muscle damage the infiltrated neutrophils and macrophages can release ROS (Leeuwenburgh & Heinecke 2001).

Many interventions have been proposed to reduce eccentric exercise-induced muscle damage and oxidative stress. Low-intensity eccentric exercise training or preconditioning exercises applied before high-intensity eccentric exercise is one of these recommendations (Lavender & Nosaka 2008). Preconditioning exercises have been applied as single or multiple bouts and the protective effect of both protocols against the high-intensity eccentric exercise-induced muscle damage have been demonstrated especially in humans (Maeo et al. 2015b, 2016, 2017). It has been reported that repeated application of low-intensity eccentric exercises that do not cause muscle damage may help to prevent the decrease in pain threshold of the muscle caused by subsequent high-intensity eccentric exercise and inhibit subcellular damage triggering ROS production and consequently prevent secondary muscle damage in rats (Munehiro et al. 2012). This protective effect has been attributed to neural adaptations, an increase in sarcomere numbers in series, extracellular matrix remodeling, and an altered inflammatory response (Hyldahl et al. 2017). Therefore, eccentric exercises have dual effect on muscle; intense eccentric exercise damages muscle, but low-load eccentric exercise protects muscle from this damage. Many studies (Chen et al. 2012, 2018, Maeo et al. 2015a). have investigated the protective effect of the one bout of low-intensity eccentric exercise on high-intensity eccentric exercise-induced muscle damage in the many aspects such as neural, molecular and biochemical. However, to the best of our knowledge no study to date has investigated the effect low-intensity eccentric exercise training on the oxidative stress and antioxidant defense markers in plasma and vastus intermedius muscle. Since the vastus intermedius is especially responsible for the eccentric contraction, in the present study this muscle tissue was evaluated (Schlagowski et al. 2016).

In the light of these information, in the present study, we tested the hypothesis that low-intensity eccentric exercise training, performed as preconditioning exercise, prevents the high-intensity eccentric exercise-induced muscle damage via decreasing oxidative stress and activating antioxidant systems. Therefore, in our study, the effect of one week of low-intensity eccentric exercise training performed before the high-intensity eccentric exercise on muscle damage and oxidative stress in the vastus intermedius muscle tissue and blood samples of the rats was investigated.

MATERIALS AND METHODS

Ethical approval

This study was approved by the Experimental Animal Ethics Committee of the Selçuk University Experimental Medicine Research and Application Center (agreement number: 2018-13). All experiments were performed based on the standard ethical guidelines (NIH Guideline for the Care and Use of Laboratory Animals).

Animals

Male Wistar albino rats (n=22, 16 weeks old; body weight, approximately 300 to 400 g) obtained from Selçuk University Experimental Animals Research and Application Center. The rats were housed in a temperature controlled room (23 ± 2 ^{o}C) with 12 hours of light, 12 hours of dark cycle

and cages where feed and water were given ad libitum, with an average of 3-4 rats per cage.

The rats were randomly divided into three experimental groups: Control (CON, n = 6), high-intensity eccentric exercise (HE, n = 8) and low-intensity eccentric exercise training plus high-intensity eccentric exercise (LET + HE, n = 8) groups. The rats in the CON group performed no exercise, but were kept on the treadmill for 5 minutes on the day of the high-intensity eccentric exercise of the other groups to eliminate stress caused by the treadmill. The rats in the HE group performed a single session high-intensity eccentric exercise protocol, which is known to induce muscle damage. The rats in the LET + HE groups were performed a low-intensity eccentric exercise protocol that did not cause muscle damage once a day for 7 days, and the same high-intensity eccentric exercise protocol as in the HE group was applied 24 hours after the last training session.

Exercise protocols

All exercise protocols were performed on a specifically designed motor-driven rodent treadmill (MAY-TME 0804, Commat Ltd., Ankara, Turkey). All rats in the exercise groups (HE and LET + HE groups) underwent a light familiarization exercise on the treadmill at 10 m/min, 0° inclinations, for - 15 min for three days. The low-intensity eccentric exercise training was performed to the LET + HE group at 10 m/min, on a - 15° incline, for 30 min/day for 7 days. The effectiveness of this exercise training protocol was demonstrated in the previous studies (Maruhashi et al. 2007, Munehiro et al. 2012). The animals in the HE and LET + HE groups were performed high-intensity eccentric exercise protocol which consists of 18 bouts of 5 min running at a speed of 20 m/min, on a 15^{o} inclination, with a 2 min rest between bouts (90 minutes of intermittent running). This muscle damaging exercise protocol was applied based on our previous study (Boz et al. 2014).

Blood and tissue sampling and storage

Twenty-four hours following the last exercise session, all animals were anesthetized by intramuscular injection of 50 mg/kg xanthine and 10 mg/kg xylazine mixture administration. Previous studies have shown that symptoms of muscle damage become apparent 12-48 hours after intense or unfamiliar eccentric exercise and peak between 24 and 72 hours (Douglas et al. 2017). Therefore, in the present study, this time-point was chosen. Blood samples were obtained directly from the left auricle by cardiac puncture. Blood samples transferred to non-additive tubes were waited for 30 min at +4 °C and then centrifuged at 3200 rpm for 30 min to obtain serum samples. Blood samples were transferred to EDTA-coated tubes were immediately centrifuged at 3200 rpm for 15 min to obtain plasma samples. Also, vastus intermedius muscle tissues of rats were immediately taken, washed with ice-cold saline, and then frozen in liquid nitrogen. All samples were stored at -80 ° C until analysis time.

Tissue homogenization

Frozen vastus intermedius muscle tissue samples were homogenized with a vibrating microbead homogenizer (FastPrep®-24 system, MP Biomedicals, Santa Ana, CA, USA) in a ratio of 1/10 ice-cold phosphate buffer for 45 sec. After homogenization, the homogenates were centrifuged at 12,000 g for 30 min at +4 °C (Hitachi Koki Co., Ltd, Himac CT 15 RE, VWR, Japan) and its supernatant was separated. All biochemical analyses were performed in these prepared homogenates.

Biochemical measurements

Serum CK and LDH activities were measured using the Abbott Architect c800 immunoassay system (Abbott Park, IL, USA) according to manufacturer's instructions. CK and LDH activities were expressed as IU/L. Serum myoglobin levels were analyzed using commercially available kits based on immunoturbidimetric methods on a Cobas Integra 400 chemistry analyzer (Roche Diagnostics, GmbH, Mannheim, Germany). Myoglobin levels were expressed as µg/L.

Plasma and vastus intermedius muscle tissue MDA (Cat No: E-EL-0060, Elabscience Biotechnology Co. Ltd., China) and GSH (Cat No: E-EL-0026, Elabscience Biotechnology Co. Ltd., China) levels and Cu-Zn SOD (Cat No: E-EL-R1424, Elabscience Biotechnology Co. Ltd., China) activities were measured using commercially available kits according to the manufacturer's recommendations. The principle of the test is based on the competitive ELISA method. The test procedure was briefly as follows; Briefly, 50 µL of standard or sample was added to each well. 50 µL of Biotinylated Ab working solution was added. It was covered and incubated at 37° C for 45 minutes. The solution in each well was aspirated, 350 µL washing buffer was added and this procedure was repeated three times. 100 µL of HRP conjugate working solution was added to each well. It was covered and incubated for 30 minutes. The solution in each well was aspirated, 350 µL washing buffer was added and this procedure was repeated five times. 90 µL of substrate reagent was added to each well. It was covered and incubated at 37 ° C for 15 minutes, protected from light. 50 µL of stop solution was added to each well. The optical density of each well was determined at once using a microplate reader (BioTek PowerWave XS, BioTek Instruments Inc., Winooski, USA) adjusted to 450 nm. The intra-assay CV of the kits was < 10%. MDA levels and SOD activities were expressed as ng/mg protein in the skeletal muscle tissue and ng/mL in the plasma. GSH levels were expressed as µg/mg protein in the skeletal muscle tissue and µg/mL in the plasma. Protein levels were determined using method of Lowry et al. (1951).

Data analysis

All data were analyzed using SPSS v. 22.0 (IBM-SPSS Inc., Chicago, IL, USA). Results were expressed as mean ± standard deviation (SD). Data normality was assessed by a Spiro-Wilk tests. The parameters providing normal distribution were tested using one-way analysis of variance. Homogeneity of variances was determined using Levene Test. Tukey Test was used in the case of variance homogeneity in multiple comparisons and Tamhane T2 test was used in case of variance heterogeneous. Kruskal-Wallis H Test was used for parameters that did not provide normal distribution. Statistical significance for all tests was accepted at $P < 0.05$.

RESULTS

Markers of muscle damage

Serum myoglobin level was higher in the HE group compared to the CON group, while it was lower in the LET + HE group compared to the HE group. (0.8 ± 0.1 µg/L, 1.9 ± 0.1 µg/L, and 1.0 ± 0.2 µg/L in the CON, HE and LET + HE groups, respectively) ($P < 0.05$). (Figure 1a). Serum CK (149.5 ± 59.7 IU/L, 724.5 ± 345.2 IU/L, and 352.3 ± 148.7 IU/L in the CON, HE and LET + HE groups, respectively) (Figure 1b), and LDH (165.3 ± 86.0 IU/L, 972.6 ± 414. 9 IU/L, and 532.4 ± 152.3 IU/L in the CON, HE and LET + HE groups, respectively) (Figure 1c) activities were higher in the HE and the LET + HE groups than the CON group. ($P < 0.05$).

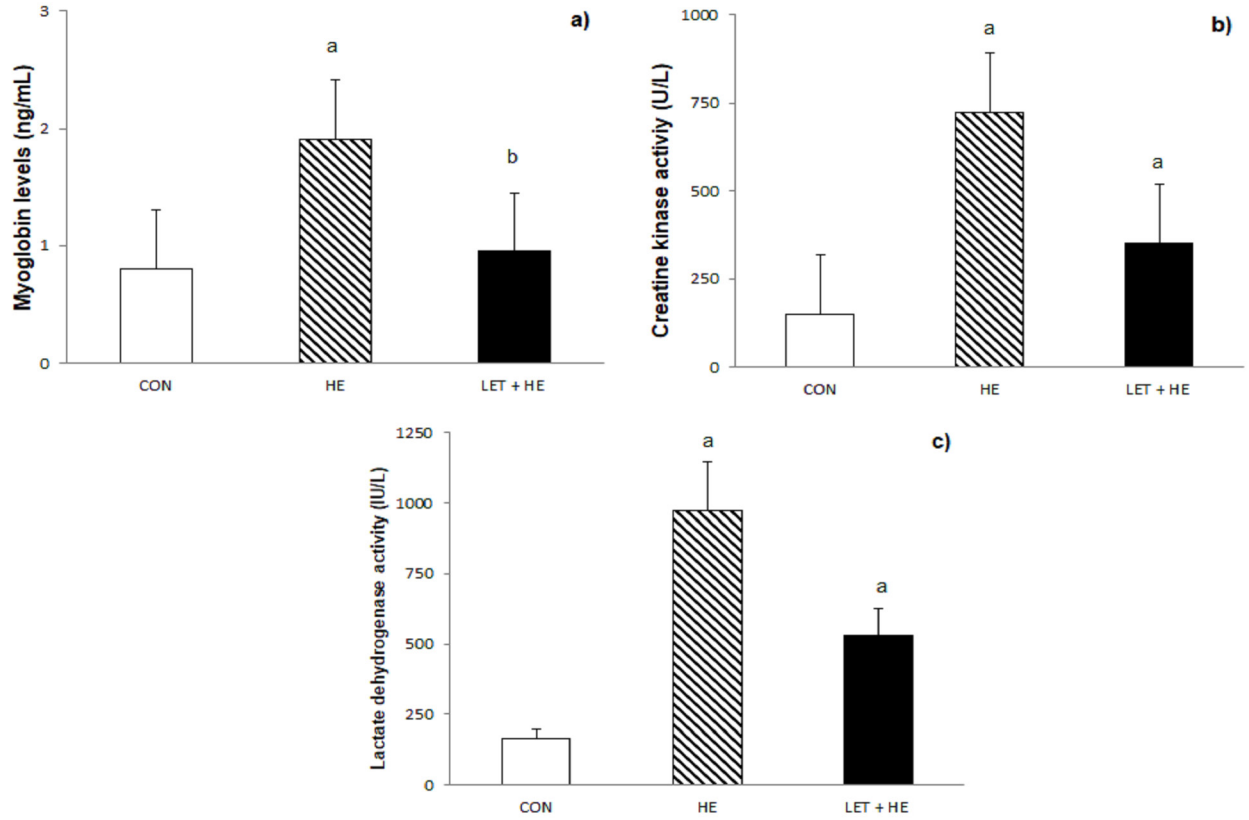

Figure 1. a) Serum myoglobin levels and b) creatine kinase and c) lactate dehydrogenase activities of the groups. Data are expressed as mean ± SD. [a]P < 0.05 compared to the CON group and [b]P < 0.05 compared to the HE group. CON: Control group, HE: High-intensity eccentric exercise group, LET + HE: Low-intensity eccentric exercise training plus high-intensity eccentric exercise groups.

Oxidative stress and antioxidants defense markers

In the vastus intermedius muscle tissue, MDA level was higher in the HE group than the CON and the LET + HE groups (17.0 ± 7.4 ng/mg protein, 28.2 ± 7.0 ng/mg protein, and 18.7 ± 6.1 ng/mg protein in the CON, HE and LET + HE groups, respectively) (P < 0.05). (Figure 2a). Plasma MDA level was higher in the HE and the LET + HE groups when compared to the CON group (147.6 ± 29.7 ng/mL, 214.3 ± 25.5 ng/mL, and 188.2 ± 22.4 ng/mL in the CON, HE and LET + HE groups, respectively) (P < 0.05) (Figure 2b).

In the vastus intermedius muscle, GSH level was higher in the LET + HE group when compared to the CON and the HE groups (6.0 ± 1.7 µg/mg protein, 6.0 ± 2.5 µg/mg protein, and 9.1 ± 2.6 µg/mg protein in the CON, HE and LET + HE groups, respectively) (Figure 3a). Plasma GSH level was higher in the LET + HE group than the HE group (76.2 ± 28.9 µg/mL, 53.1 ± 24.1 µg/mL, and 84.9 ± 19.0 µg/mL in the CON, HE and LET + HE groups, respectively) (Figure 3b). However, there was no significant difference between the CON and the HE groups in the vastus intermedius muscle and the plasma (P > 0.05).

In the vastus intermedius muscle, SOD activity was higher in the LET + HE group when compared to the CON group (4.8 ± 2.9 ng/mg protein, 6.2 ± 2.3 ng/mg protein, and 8.6 ± 2.6 ng/mg protein in the CON, HE and LET + HE groups, respectively) (P < 0.05), but were not different between the CON and HE groups (P> 0.05)

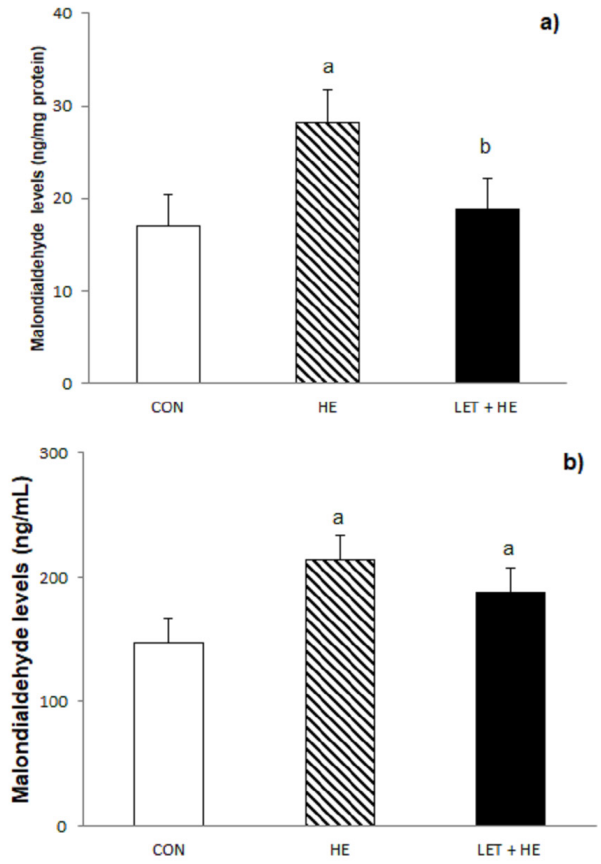

Figure 2. a) Vastus intermedius muscle and b) plasma malondialdehyde levels of the groups. Data are expressed as mean ± SD. [a]P < 0.05 compared to the CON group and [b]P < 0.05 compared to the HE group. CON: Control group, HE: High-intensity eccentric exercise group, LET + HE: Low-intensity eccentric exercise training plus high-intensity eccentric exercise groups.

(Figure 4a). There was no significant difference in plasma SOD activity between the groups (0.8 ± 0.3 ng/mL, 0.7 ± 0.3 ng/mL, and 0. 8 ± 0.2 ng/mL in the CON, HE and LET + HE groups, respectively) (P > 0.05) (Figure 4b).

DISCUSSION

In the present study, we examined the effect of low-intensity eccentric exercise training on high-intensity eccentric exercise-induced muscle damage and oxidative stress in the rats. The findings suggest that repeated series of low-intensity eccentric exercises may prevent muscle damage caused by high-intensity eccentric exercise in rats. Another important finding is that low-intensity eccentric exercise training decreased high-intensity eccentric exercise-induced oxidative stress especially in the vastus intermedius muscle via improving the enzymatic and non-enzymatic antioxidant defense systems. To the best of our knowledge, the present study is the first to investigate the effect of low-intensity eccentric exercise training on high-intensity eccentric exercise-induced muscle damage, oxidative damage and antioxidant defense in the vastus intermedius muscle.

The most common results of high-intensity eccentric exercise include elevated levels and activities of the muscle damage biomarkers such as myoglobin, CK and LDH in the circulation (Isner-Horobeti et al. 2014, Boz et al. 2014). In our study, serum myoglobin levels and, CK and LDH activities increased after high-intensity eccentric exercise. Low-intensity eccentric exercise training for 7 days performed before the high-intensity eccentric exercise prevents the elevation in the myoglobin level, but CK and LDH activities remained significantly higher than in the control group, although not as high as the high-intensity group. In consistent with our findings Maruhashi et al. (2007) reported that low-load eccentric exercise training prevents intense eccentric exercise-induced muscle damage in rats. Additionally, in a recent study, Yamada et al. (2018) reported that preconditioning contractions prevent muscle damage induced by damaging eccentric contractions in rats.

In this study, it was shown that 7 days of low-intensity eccentric exercise training had a protective effect against muscle damage caused by high-intensity eccentric exercise. In the current literature, low-intensity eccentric exercise training protocols have been applied in humans

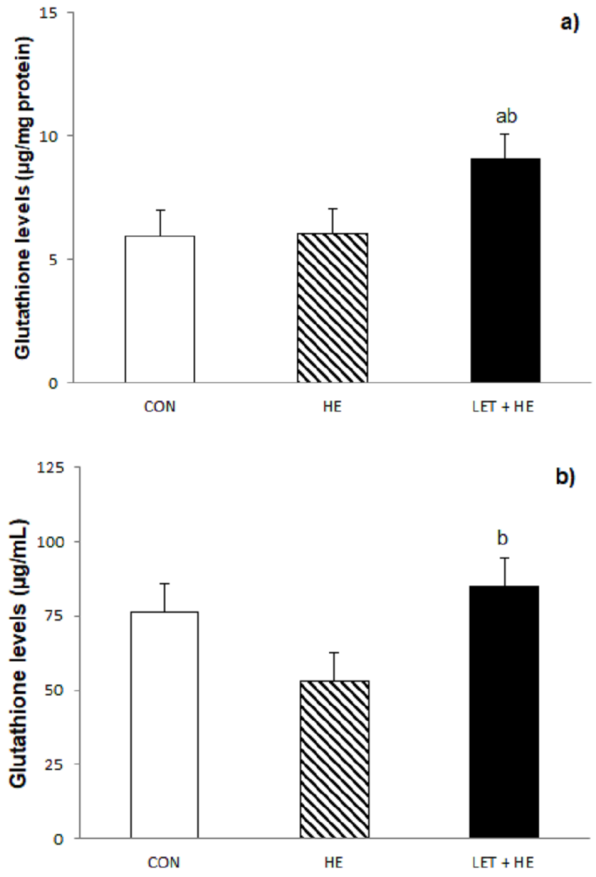

Figure 3. a) Vastus intermedius muscle and b) plasma glutathione levels of the groups. Data are expressed as mean ± SD. [a]P < 0.05 compared to the CON group and [b]P < 0.05 compared to the HE group. CON: Control group, HE: High-intensity eccentric exercise group, LET + HE: Low-intensity eccentric exercise training plus high-intensity eccentric exercise groups.

and experimental animals for different durations ranging from 2 days to 4 weeks (Fernandez-Gonzalo et al. 2011, Chen et al. 2012, Munehiro et al. 2012, Lin et al. 2015). However, although there are studies showing that the protective effect occurs after 2 days, the protective effect has generally been demonstrated in protocols of 5 days and longer. Therefore, in this study we used a 7-day low-intensity eccentric exercise training protocol based on previous studies (Maruhashi et al. 2007, Munehiro et al. 2012).

In the present study, MDA, GSH and SOD were evaluated as markers of the oxidative stress and antioxidant defense system in the blood, and vastus intermedius muscle tissue. Aerobic metabolism during exercise and the inflammatory processes in damaged muscle can both produce ROS (Best et al. 1999). MDA is the product of peroxidation of lipids and indirectly reflects the degree of ROS in membrane and the increased MDA levels may play an important role in the development and progression of exercise-induced muscle damage (Liu et al. 2013). In the present study, after high-intensity eccentric exercise, MDA levels increased in the vastus intermedius muscle and plasma. It has been demonstrated that MDA levels are increased (Cabral de Oliveira et al. 2001, Close et al. 2005) or unchanged (You et al. 2005, Molnar et al. 2006, Boz et al. 2014) in response to high-intensity eccentric exercise in the blood and the skeletal muscle. In the present study, low-intensity eccentric exercise training prevented an increase in MDA levels in the vastus intermedius muscle, while the plasma MDA level remained unchanged. This may be due to low-intensity eccentric exercise activating or strengthening the antioxidant defense system especially in the muscle tissue. In contrast to our findings Maruhashi et al. (2007) reported that low-load eccentric exercise training prevents intense eccentric exercise-induced muscle damage independent from the oxidative stress in rats. The differences between the results might be explained by the differences in the exercise protocols, sampling time, and analyzed muscle fiber type.

GSH is considered to be the most important and abundant endogenous non-enzymatic antioxidant and protects cells from singlet oxygen, hydroxyl radicals and superoxide radical damage (Espinosa-Diez et al. 2015). In the present study, higher GSH levels in the vastus intermedius muscle and the plasma of the LTE + HE group suggests that low-intensity

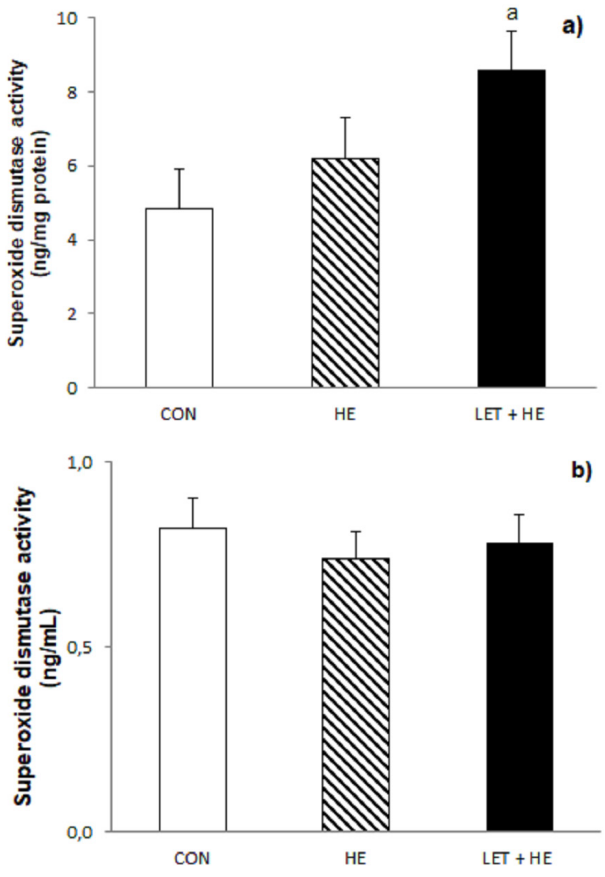

Figure 4. a) Vastus intermedius muscle and b) plasma superoxide dismutase activities of the groups. Data are expressed as mean ± SD. [a]P < 0.05 compared to the CON group. CON: Control group, HE: High-intensity eccentric exercise group, LET + HE: Low-intensity eccentric exercise training plus high-intensity eccentric exercise groups.

eccentric exercise training upregulate non-enzymatic antioxidant system and therefore preventing the impairment of the antioxidant defense following the high-intensity eccentric exercise. In this sense, regular exercise has been suggested to stimulate adaptations leading to higher antioxidant capacity and, consequently, more efficient neutralization of ROS, due to repeated activation of antioxidant genes and proteins (Steinbacher & Eckl 2015). In the current literatures, there are contradictory results about the GSH response to the high-intensity eccentric exercise. Goldfarb et al. (2004) showed that oxidized glutathione (GSSG) levels increase immediately and 2 h after the exercise. However, Lee et al. (2002) and You et al. (2005) showed that GSSG/GSH ratio tended to increase, but Close et al. (2005) reported that GSH levels tended to decrease at several time periods following exercise. In the previous study (Boz et al. 2014), we have showed that plasma and gastrocnemius muscle tissue GSH levels did not change immediately after the high-intensity eccentric exercise. The differences between the results might be explained by the differences in the exercise protocols, sampling time, and analyzed muscle fiber type.

SOD is one of the most important enzymatic antioxidants and is the first step of the antioxidant defense system. It can convert superoxide radicals to hydrogen peroxide. It has been reported that SOD effectively protects from muscular oxidative stress caused by exercise (Finaud et al. 2006). In the present study, higher SOD activity in the vastus intermedius muscle tissue of the LTE + HE group thought that low-intensity eccentric exercise training enhances the antioxidant defense by increasing the enzymatic SOD activity and thus protects against the oxidative stress caused by the high-intensity eccentric exercise. There are limited and contradictory studies (Molnar et al. 2006, Zhao et al. 2013, Boz et al. 2014) investigating the effects of single bout of high-intensity eccentric and low-intensity eccentric exercise training on enzymatic antioxidants such as SOD, catalase (CAT), and glutathione peroxidase (GPx). Zhao et al. (2013) reported that SOD1 mRNA expression increased but SOD3 mRNA expression decreased at 30 min after the high-intensity eccentric exercise in the rectus femoris muscle of the rats. In contrast, Molnar et al. (2006) and Boz et al. (2014) showed that there was no effect of high-intensity eccentric exercise on SOD activity immediately after the

exercise in the skeletal muscle and the plasma. Additionally, Yamada et al. (2018) showed that low-intensity eccentric exercises as a model of preconditioning contractions increase the expression of antioxidant enzymes SOD and CAT in the rat medial gastrocnemius muscle tissue. In this study, SOD activity increased in muscle tissue with low-intensity eccentric exercise training, while there was no significant difference in plasma. This indicates that SOD may play a more prominent role in intramuscular antioxidant response or that intramuscular SOD changes are not reflected in plasma as a result of the training protocol we apply.

In this study, the oxidative stress marker was lower and antioxidant defense markers were higher in the vastus intermedius muscle of the LET + HE group. However, it is not yet fully explained whether the improved redox state causes less damage or whether less damage improves the redox state. Consistent with our findings, Yamada et al. (2018) reported that preconditioning contractions can improve recovery after damaging eccentric contractions by reducing myeloperoxidase-mediated ROS production. In contrast, Maruhashi et al. (2007) and Silva et al. (2013) reported that low-load eccentric exercise training prevents intense eccentric exercise-induced muscle damage independent from the oxidative stress in rats. The results of this study suggest that low-intensity eccentric exercise training may improve oxidative stress and antioxidant defense, resulting in less muscle damage following high-intensity eccentric exercise.

The mechanism of how the changes in the oxidative stress responses reduce the magnitude of muscle damage is unclear. Radical production, induced by eccentric exercise, can adversely affect skeletal muscle fibers and affect the magnitude of muscle damage. Best et al. (1999) reported that observed damage in artificially stimulated and stretched rabbit muscle was associated with delayed ROS production accompanied by adaptive responses of ROS scavenging enzymes. Therefore, as in this study activation of the both enzymatic and non-enzymatic antioxidant defense systems decrease oxidative stress response and may reduce magnitude of muscle damage.

This study has several weaknesses that should be underlined. First weakness is the lack of serial blood sampling in the different time intervals to observe the time-dependent changes in the blood and tissue samples. Second weakness is we were unable to perform more detailed oxidative stress and antioxidant defense system analysis such as total oxidant and antioxidant status or protein carbonyls. Third weakness is we could not analyze the effect of moderate-intensity eccentric exercise in different periods such as 2 or 7 days before the high-intensity eccentric exercise to expand the study. Another weakness is the lack of molecular and histological techniques due to the technical difficulties.

As a result, low-intensity eccentric exercise training performed before the high-intensity eccentric exercise protective effect against muscle damage, in particular, decreases oxidative stress formation especially in the vastus intermedius muscle and increases the SOD and GSH. More detailed research is needed to clarify the mechanism of action of the low-intensity eccentric exercise in both animals and humans.

Acknowledgments

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