Experimental Study / *Deneysel Çalışma*

The Effect of Regular Training with Vitamin E Supplementation on the Thioredoxine System in Rats

E Vitamini ve Düzenli Antrenmanın Sıçanların Tiyoredoksin Sistemi Üzerine Olan Etkisi

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Objectives: The aim of this study was to investigate the effects of both regular swimming training and vitamin E supplementation on the thioredoxin system, one of the key player in defense against oxidative stress. In order to examine the effects of regular physical exercise on the antioxidant defenses of tissues and on their susceptibility to damage induced by exercise, the activity of thioredoxin reductase (TrxR) have been determined.

Materials and Methods: Four groups of male rats were used. Two groups were trained to swim for nine weeks. One trained and one sedentary group received vitamin E supplementation for four days a week.

Results: Thiobarbituric acid-reacting substance (TBARS) levels of the trained group significantly decreased ($p<0.01$) in the liver and the brain. Meanwhile, both regular exercise and vitamin E supplementation caused increases ($p<0.001$) in TrxR activities in all tissues.

Conclusion: These results suggest that increased activity of TrxR is an important oxidative response of the thioredoxin system and plays an important protection role against protein damage. It is also likely that vitamin E depletion in oxidative stress may require recycling process of this molecule in relation to TrxR, supporting with the TrxR activities of tissues in both trained and vitamin E-supplemented group.

Key words: Thioredoxine; TrxR; TBARS; vitamin E; regular training.

Amaç: Bu çalışmanın amacı düzenli yüzme antrenmanı ve E vitamini desteğinin, oksidan strese karşı savunmada anahtar rol oynayan tiyoredoksin sistem üzerine olan etkilerini araştırmaktı. Düzenli fiziksel egzersizin dokuların (karaciğer, beyin ve akciğer) antioksidan savunmaları ile egzersiz tarafından tetiklenen duyarlılıkları üzerine etkilerini belirlemek için tiyoredoksin redüktaz (TrxR) aktivitesi araştırıldı.

Gereçler ve Yöntemler: Dört grup erkek sıçan kullanıldı. İki gruba dokuz hafta süreyle yüzme antrenmanı yaptırıldı. Antrene ve sedanter birer gruba haftada dört gün E vitamini enjeksiyonu gerçekleştirildi.

Bulgular: Antrene grupların tiobarbitürik asit reaktif ürün (TBARS) düzeyleri karaciğer ve beyinde anlamlı olarak düşüktü ($p<0.01$). Düzenli egzersiz ve E vitamini desteği analiz yapılan tüm dokulardaki TrxR aktivitelerinde anlamlı artışa ($p<0.001$) sebep oldu.

Sonuç: Bu sonuçlar, artan TrxR aktivitesinin tiyoredoksin sistemin önemli bir oksidatif cevabı olduğunu ve protein hasarı karşısında önemli bir koruyucu rol oynadığını ortaya koymaktadır. E vitamini alan antrenman grubundaki dokuların TrxR aktivitesi artmış olup, muhtemelen oksidatif stres sırasındaki E vitamini eksikliği bu molekülün TrxR ile ilişkili geri dönüşüm sürecinin desteklenmesini gerektirebilir.

Anahtar sözcükler: Tiyoredoksin; TrxR; TBARS; E vitamini; düzenli antrenman.

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Exercise can enhance reactive oxygen species (ROS) production in various tissues depending on the tissue oxygen consumption rate.[1] The increased oxygen intake and elevated metabolic rate during physical exercise lead to excessive ROS production, related to the intensity and/or the duration of exercise.[2] As it is known that physical activity is a source of stress, and regular training, a chronic physical stress, is able to release adaptations in response to a higher production of ROS.[3] These adaptations are related to several systems, among which the key player is the thioredoxin system, composed by thioredoxin (Trx) and thioredoxin reductase (TrxR).[4,5]

The thioredoxin system protects the cell against oxidative stress by scavenging reactive oxygen species through a variety of direct and indirect mechanisms. TrxR, a member of this family, is a seleno-protein and maintains Trx in a reduced state by catalyzing the NADPH-dependent reduction of Trx disulfide.[6] Unlike Trx, TrxR has broad substrate specificity and can reduce non-disulfide substrates including lipid hydroxides.[7]

Mammalian TrxR exists in three different forms: TR1, TR3 and TGR. TGR is regarded as the novel form that functions as a thioredoxin and glutathione reductase.[8,9] The expression of the thioredoxin system induced by ROS, produces elevated levels of Trx and TrxR as an oxidative response.[10,11]

Vitamin E is well accepted as the first line of defense against lipid peroxidation, protecting polyunsaturated fatty acids in cell membranes through its free radical quenching activity in bio-membranes at an early stage of free radical attack.[12,13] Mild, chronic oxidative stress caused by exercising probably alters the antioxidant systems by depleting cellular stores of vitamin E. However, physiological levels of tissue vitamin E provide adequate protection against exercise-induced ROS generation.[14,15]

Vitamin E cannot be synthesized by most mammals and humans; therefore is required from the diet.[16] However, dosage of the supplementation should be carefully considered since no study has either confirmed or denied a potential effect of overdose and side effects. In this study, we focused on the antioxidant defense mechanism of the thioredoxin system, mainly the enzyme TrxR against the training process and to evaluate the possible effects of vitamin E supplementation on the thioredoxin system, in trained and sedentary rats.

MATERIALS AND METHODS

Animals

Male Wistar Albino rats, 3 months of age with an average weight of 150 to 190 g were used. The rats were randomly divided into four groups: Two of the groups were subjected to swimming training while the other two were the sedentary groups. Group 1 consisted of sedentary rats (n=7), Group 2 vitamin E-supplemented sedentary rats (n=8), Group 3 trained rats (n=8), Group 4 vitamin E-supplemented trained rats (n=7). These animals were housed in conventional wire-mesh cages, four rats per cage, in a room with the temperature regulated at 21±1°C, humidity 45-50% and in daily light/dark cycle (12 h). All rats were given ad libitum access to food, and tap water by drinking bottle throughout the course of the experiment. They were fed a standard laboratory diet and received human care according to the criteria outlined in the 'Guide for the Care and Use of Laboratory animals' prepared by the National Academy of Science and published by the National Institutes of Health.[17]

Exercise

The exercise groups of rats were submitted to swim for five minutes a day and speed of exercise increased five minutes per day. In the second week, they were submitted to swim for 30 minutes a day and four days a week which lasted for nine weeks. Rats swam individually in a 21 cylindrical glass beaker filled with water ≈ 30 cm deep. The beakers were submerged in a thermostatic water bath set at 30°C. The fur of the rat was washed with liquid soap prior to swimming and air bubbles trapped in the fur were removed periodically to reduce buoyancy and ensure the imposed work load. Sedentary rats remained in their cages throughout the study.

Supplementation

During the experimental procedure, one of the trained groups and one of the sedentary groups were treated with vitamin E (dl-alpha tocopheril acetate, Evigen, Aksu Pharma, Turkey, 50 mg/kg body wt/day) injected intra-peritoneal for four days a week, whereas the other groups were injected the same amount of saline only. All injections were performed three hours before swimming exercise. The effective dosage (50 mg/kg body wt/day) was preferred on the basis of the concentration capable of providing a larger antioxidant protective margin.[18]

Tissue preparation

Nine weeks later, all rats, anesthetized with sodium pentothal (Abbott, Campoverde di Aprilia, Italy, 100 mg/kg) 48 hours after the last session. The tissues were removed from all animals in the same order: lung, liver, brain. The tissues were immediately placed in ice cold buffer, kept on ice and immediately transferred to a –80°C freezer where they were kept until analysis. Before the assay, tissue samples were homogenized in ice-cold phosphate (including EDTA, triton-X, butylated hydroxytoluene) buffer. Then homogenates were centrifuged at 2000xg for ten minutes and supernatants were used in the biochemical analysis. Furthermore, rat tissue samples were treated with a protease inhibitor cocktail containing 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pepstatinA, E-64, bestatin, leupeptin, and aprotinin for the measurement of TrxR activity. This cocktail added to the extract buffer at 1:100 dilutions to prevent unwanted proteolysis of the sample.

Table 1. TBARS levels of rats (nmol/g protein)

Tissues	Group 1 Sedentary	Group 2 Sedentary + vitamin E	Group 3 Trained	Group 4 Trained + vitamin E
Liver	119.7±14.9	84.1±11.4	108±11[aa]	91.2±7.3[b]
Lung	67.9±7.4	52.2±8.0	71±8.1	50.3±8.7
Brain	54.7±6.7	48.1±7.2	47.8±7.7[aa]	45.2±8.1

Values are means ± SE. (aa): Group 3 vs Group 1 ($p<0.01$); (b): Group 4 vs Group 2 ($p<0.05$).

Measurement

Measurement of lipid peroxidation (LPO) levels. The thiobarbituric acid-reacting substances (TBARS) in the tissues were determined as a marker of LPO by the spectrophotometer, as described by Stocks and Dormandy.[19]

Measurement of protein oxidation. Protein oxidation was determined spectrophotometrically by measuring protein carbonyls with 2,4 dinitrophenyl hydrazine.[20]

Measurement of thioredoxine reductase (TrxR) activity. TrxR activity was determined spectrophotometrically by using the TrxR assay kit (Sigma, Saint-Louis, Missouri, USA). The assay is based on the reduction of 5,5'-dithiobis (2-nitrobenzoic) acid (DTNB) in the presence of NADPH.[7]

Statistical analysis

The results were expressed as mean ± SE, and statistical analyses were done by using one-way ANOVA with Bonferroni's post hoc tests. $p<0.05$ was accepted as significant.

RESULTS

Table 1 shows TBARS levels as an index of lipid peroxidation. TBARS levels of Group 3 significantly decreased in the liver and the brain ($p<0.01$) when compared to Group 1. The liver tissue TBARS levels of Group 4 were significantly higher ($p<0.05$) than Group 2. No statistically significant changes were observed in the lung.

As shown in Table 2, mean protein carbonyl levels of all tissues were increased in Group 3 when compared to Group 1. Both liver and lung tissues protein carbonyl levels of Group 4 were higher than Group 2 whereas the brain tissue was lower.

Table 3. TrxR activities of rats (ΔAbs412/min/mg protein)

Tissues	Group 1 Sedentary	Group 2 Sedentary + vitamin E	Group 3 Trained	Group 4 Trained + vitamin E
Liver	0.347±0.104	0.392±0.123	0.461±0.181[aaa]	0.532±0.132[bbb]
Lung	0.262±0.089	0.287±0.091	0.312±0.092[aaa]	0.413±0.102[bbb]
Brain	0.447±0.199	0.472±0.161	0.579±0.216[aaa]	0.671±0.111[bbb]

Values are means ± SE. (aaa): Group 3 vs Group 1 ($p<0.001$); (bbb): Group 4 vs Group 2 ($p<0.001$).

Table 2. Protein carbonyl levels of rats (nmol/mg protein)

Tissues	Group 1 Sedentary	Group 2 Sedentary + vitamin E	Group 3 Trained	Group 4 Trained + vitamin E
Liver	2.91±0.72	2.26±0.68	3.0±0.92	2.42±0.71
Lung	2.44±0.54	2.38±0.67	2.54±0.59	2.41±0.61
Brain	1.81±0.73	1.76±0.21	1.87±0.88	1.74±0.27

Values are means ± SE.

The activities of TrxR in all tissues were summarized in Table 3. It was remarkable that TrxR activities of all tissues were significantly elevated ($p<0.001$) in both Group 3 and Group 4 when compared to Group 1 and Group 2, respectively.

DISCUSSION

The thioredoxin system is involved in many cellular defenses against oxidative stress, thus it is logical that the expression of the thioredoxin system is also induced in response to oxidative stress.[21]

Further evidence for the role of the thioredoxin system in defense against oxidative stress is found in the induction of thioredoxin and the TrxR gene expression in the lungs of newborn primates. At birth, the sudden exposure of the lung (from the previous relatively anaerobic, fetal environment) to oxygen produces acute oxidative stress resulting in elevated levels of thioredoxin, TrxR mRNA and protein.[22]

Recent findings in conjunction with extensive research suggest that regulation of the thioredoxin and TrxR gene expression involves extremely complex mechanisms that are designed to respond to oxidative stress. On the other hand, there have been many reports showing that exercise causes increases in oxidative damage markers,[23] effects on mitochondrial function.[24] Therefore we have focused on how the activity of TrxR in various tissues is affected in trained rats which had performed regular swimming exercise and whether vitamin E supplementation has an effect on the thioredoxin system.

Several studies have shown the antioxidant effect of vitamin E in different types of exercise models in which oxygen metabolism and consequently free radical production are greatly accelerated.[25] More rapid depletion of vitamin E from liver and muscle have been observed in rats undergoing endurance training than in sedentary controls at similar vitamin E intakes.[26] Certainly, the depletion of tissue vitamin E store enhanced LPO and mitochondrial dysfunction in exhaustively exercised rats.[27] Because the primary antioxidant vitamin E is consumed by the tissues during increased physical exercise, results suggest that there is an increased vitamin E requirement during endurance training.[28] Vitamin E copes with free radicals and form vitamin E semiqui-

none, which in turn can be reduced back to vitamin E. This fact points out the need for compounds that can efficiently recycle vitamin E.[29] In fact, since ascorbic acid recycles vitamin E and mammalian TrxR reduces dehydroascorbic acid to ascorbic acid, TrxR may play an important role for the total vitamin E antioxidant function.

In this study, both regular exercise and vitamin E supplementation caused a significant increase in the tissue TrxR activities in comparison to those of both sedentary and only vitamin E-supplemented groups. The increase in the activity of this enzyme may be dependent on several factors. As it is probable that ascorbate reduces oxidized vitamin E by intermediary one-electron steps,[30] there is a potential linkage between the ascorbate/ vitamin E and Trx-TrxR systems, both of which derive their reducing activity from TrxR and NADPH.

Although our results may be contrary to the hypothesis that supplying of large amounts of antioxidants externally may require much less internal complementary antioxidants[31] and therefore there is much less antioxidant repair for thioredoxin systems, we assume that the increase in the activity of TrxR may be dependent on the continuous recycling of vitamin E by reducing oxidized form of this molecule and also repairing proteins by means of reducing protein thiols to protect cellular components against exercise-induced oxidative damage.

Thus supporting our hypothesis, the protein carbonyl content of the tissues increased in regular exercised rats as reflecting protein oxidation. To maintain low cellular redox state, TrxR activity also increased and vitamin E supplementation may contribute to the maintenance of enzyme activity to support continuous recycling of this vitamin. Meanwhile, increased activity of this enzyme protects protein and other cellular components against oxidative damage.

The present study also demonstrated that TBARS levels significantly decreased in the liver and the brain except the lung in which an increase was observed. It can be explained that this organ is the first station faced to O_2 directly and there is a high increase in the respiration load in the exercise process.[32]

In addition, there was a significant elevation on TBARS level of liver in Group 4. This finding can be accounted for exercise-induced pro-oxidants that exceeded the level which can be neutralized by the existing antioxidant systems of the rats with vitamin E supplementation due to the applied regular training of moderate intensity.

Exercise is claimed to be an important parameter increasing LPO.[33] Besides, during acute and chronic exercise protocols, in experiments carried out both on humans and animals, it is considered that different tissue and blood LPO levels remained unchanged,[34] even decreased.[35] Variations in the LPO due to regular endurance training are contradictory, as well. The differences among organs may be dependent on several homeostatic factors, such as oxygen consumption, susceptibility to oxidants and to antioxidant enzyme activation, antioxidant levels, and other repair systems.

Consequently, the response of the thioredoxin system to oxidative stress creates increased TrxR activity in regular training. It has long been known that vitamin E is the major fat-soluble antioxidant responsible for protection cells from oxidative damage and TrxR may serve as a link existing in the recycling of vitamin E which is crucial in the substitution of depleted vitamin E body stores.

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