Effects of treadmill exercise test on oxidative/antioxidative parameters and DNA damage

Tredmil efor testinin oksidan/antioksidan parametreler ve DNA hasarı üzerine etkisi

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ABSTRACT

Objective: We investigated the acute effects of treadmill exercise test (TET) on total peroxide, total antioxidant capacity (TAC), oxidative stress index (OSI) and DNA damage levels in voluntary and untrained healthy subjects.

Methods: A total of 113 untrained healthy subjects were included in the study. All subjects maintained a similar diet and physical activity for a week before the test. Blood samples were obtained before and after TET. Total peroxide, TAC, vitamin C and DNA damage were measured. The DNA damage was analyzed by using the Comet assay and OSI was calculated using total peroxide and TAC values.

Results: Treadmill exercise test leads to the increase of total peroxide (12 ± 3 μmol H_2O_2/L to 14 ± 3 μmol H_2O_2/L, $p<0.001$), OSI (0.72 ± 0.18 AU to 0.81 ± 0.22 AU, $p<0.001$), and to the decrease of TAC (1.78 ± 0.16 mmol Trolox Eq./L to 1.72 ± 0.15 mmol Trolox Eq./L, $p<0.001$) and vitamin C levels (98 ± 4.2 μmol/L to 95 ± 3.4 μmol/L, $p<0.001$). There was not significant difference in DNA damage.

Conclusion: Our findings demonstrate that TET increases oxidants, decreases TAC and vitamin C namely, the balance shift towards oxidative side, but this stress is not enough to produce DNA damage. *(Anadolu Kardiyol Derg 2006; 6: 135-40)*

Key words: Antioxidants, treadmill exercise test, DNA damage, oxidants

ÖZET

Amaç: Bu çalışmada gönüllü ve antrenmansız sağlıklı kişilerde tredmil efor testinin total peroxid, total antioksidan kapasite (TAK), oksidatif stres indeksi (OSİ) ve DNA hasarı üzerine olan etkisi araştırıldı.

Yöntemler: Toplam 113 sağlıklı antrenmansız kişi çalışmaya dahil edildi. Bütün kişiler tredmil testinde bir hafta öncesinde benzer diyet ve fiziki aktivite önerildi. Efor testine önce ve hemen sonra kan örnekleri alındı. Total peroxid, TAK, vitamin C ve DNA hasarı ölçümleri yapıldı. DNA hasarı Comet değerlendirme yöntemi ile OSİ'de total peroxidin TAK'a bölünmesi ile hesaplandı.

Bulgular: Tredmil testi sonucunda öncesine göre total peroxid (12 ± 3 μmol H_2O_2/L'den 14 ± 3 μmol H_2O_22/L'ye, $p<0.001$) ve OSİ (0.72 ± 0.18 AU'ten 0.81 ± 0.22 AU'e, $p<0.001$) değerlerinde anlamlı artış ile TAK (1.78 ± 0.16 mmol Trolox Eq./L'den 1.72 ± 0.15 mmol Trolox Eq./L'ye, $p<0.001$) ve vitamin C değerlerinde (98 ± 4.2 μmol/L'den 95 ± 3.4 μmol/L'ye, $p<0.001$) belirgin azalma saptandı. DNA hasarında öncesi ve sonrasına göre anlamlı bir değişme izlenmedi.

Sonuç: Bu çalışma ile tredmil efor testi sonucunda oksidanların artması, TAK ve vitamin C başta olmak üzere antioksidanları azalması sonucunda dengenin oksidatif strese doğru kaydığı fakat bu stresin DNA hasarı oluşturması için yeterli olmadığını düşündürmektedir. *(Anadolu Kardiyol Derg 2006; 6: 135-40)*

Anahtar kelimeler: Antioksidanlar, tredmil egzersiz testi, DNA hasarı, oksidanlar

Introduction

Oxidants such as superoxide, hydrogen peroxide and hydroxyl radicals are produced in metabolic and physiological processes, and harmful oxidative reactions may occur in organisms. Antioxidant molecules prevent and/or inhibit these harmful reactions (1,2). Proteins constitute the main antioxidant component of serum. The uric acid, bilirubin, vitamin C, Trolox, and polyphenols have also antioxidative effects. Plasma concentrations of different antioxidants can be measured in laboratories separately, but the measurements are time-consuming, labor-intensive, costly, and they require complicated techniques. Since the measurement of different antioxidant molecules separately is not practical and antioxidant effects of them are additive, total antioxidant capacity (TAC) of a sample is measured (3, 4). Oxidative stress is an imbalance between the production of free radicals that contain unpaired electrons, which increase the chemical reactivity, and antioxidant defenses buffering the oxidative damages (5). It causes changes to biological molecules, and these changes accumulate over time in the biological structures (6,7), which may cause molecular damage to cellular and tissue structures (5). It was also known that plasma antioxidant capa-

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Note: This study was presented at the 1st Congress of Clinic Vascular Biology, Antalya, Turkey, 6-9-April, 2005.

city decreases and oxidative/antioxidative balance shifted to oxidative side in patients with coronary artery disease (8,9).

The treadmill exercise test (TET) is one of the most frequently used noninvasive tests to assess the patients with suspected or proven coronary artery disease (10). Exercise cause increases in oxygen consumption, production of reactive oxygen species (ROS), and these lead to oxidative stress (11).

DNA damage is caused by multiple endogenous and exogenous factors such as oxidative stress, age, smoking, hypertension, hyperlipidemia and diabetes mellitus (12). Antioxidant systems prevent the damage of DNA (13). It has been shown that excessive exercise induces DNA damage in peripheral leukocytes (14).

Previous studies have indicated that antioxidants increase and plasma lipid peroxide levels decrease during training exercise (15-17). The published reports support that chronic exercise increases antioxidant defenses (18). However, it is not completely known whether there is any change in TAC, oxidative stress index (OSI) and total peroxide during short exercise such as TET.

Most of the previous studies on exercise and oxidative stress have used high or moderate intensity exercise regimens, including exhaustive exercise (19). The acute effects of TET on oxidative/antioxidative system and DNA damage are not well known. In this study, we investigated acute effects of TET on serum oxidant/antioxidant balance and DNA damage in voluntary subjects with suspected coronary artery disease.

Materials and Methods

Subjects

One hundred thirteen consecutive voluntary subjects with typical angina or angina-like symptoms being referred for evaluation of suspected coronary artery disease (64 males, 49 females, mean age: 46 ± 11 years) were included in the study. In addition, all participants selected were sedentary (i.e., were not currently participating in regular endurance exercises such as walking, jogging, cycling, dance aerobics, swimming, etc., and had not done so for the previous 12 months). Subjects with hypertension, diabetes mellitus, dyslipidemia, acute or chronic inflammatory disease, immunological disease, history or presence of neoplastic disease, alcohol consumption greater than 40 g per day, or medication use, including mineral or vitamin supplements, smoking and body mass index >30 kg/m^2 were excluded. In addition, the individuals with angina or any other cardiac or pulmonary symptoms potentially limiting exercise performance and not reaching target heart rate (THR) were excluded as well. All participants maintained a similar diet and physical activity for a week before the test. No subjects were receiving antioxidant therapy. Body mass index was computed as weight divided by height squared (kg/m2). Waist circumference was measured before TET. Informed consent was obtained from all subjects after a full explanation of the study.

Exercise Treadmill Test

All subjects underwent treadmill exercise testing using the modified Bruce protocol. Before testing, all subjects were instructed to not eat, drink, or smoke for 3 hours before the testing. Angina, fatigue, diagnostic ST-segment depression, or persistent arrhythmias were considered reasons for discontinuing the exercise test. The ST-segment level was measured 60 ms after the J point in all 12 electrocardiogram (ECG) leads. The heart rate, ECG, and blood pressure were recorded at the onset and immediately after exercise. The target heart rate (THR) was established for each participant as 75%of their maximum heart rate (HRmax) calculated by the formula HRmax =0.75 (220-age (years). Participants selected a comfortable speed and walked on the treadmill at an elevation of 5% until their heart rate reached THR and then continued to walk for 60 s after reaching THR. A continuous 12-lead ECG strip was recorded during the test. Definitive positive criteria for exercise testing were defined as: horizontal or downsloping ST segment depression ≥1 ms, or upsloping ST segment depression, ≥2 mm in any lead, present within the first 2 minutes of the recovery period.

Samples

Blood samples were withdrawn into heparinized tubes from a cubital vein before and immediately after TET to measure oxidative, antioxidative parameters and DNA damage. One milliliter of blood was pipetted into another tube immediately to measure DNA damage. Remaining blood was centrifuged at 3000 rpm for 10 minutes for plasma separation. Plasma samples were stored at -80°C until analysis of TAC, total peroxide and vitamin C.

Measurement of total antioxidant capacity and vitamin C level

The TAC of plasma, taken before the angiographic procedure, was determined using a novel automated measurement method, developed by Erel (3). In this method, hydroxyl radical, which is the most potent biological radical, is produced. In the assay, ferrous ion solution, which is present in the Reagent 1 is mixed by hydrogen peroxide, which is present in the Reagent 2. The sequentially produced radicals such as brown colored dianisidinyl radical cation, produced by the hydroxyl radical, are also potent radicals. In this assay, antioxidative effect of the sample against the potent free radical reactions, which is initiated by the produced hydroxyl radical, is measured. The assay has got excellent precision values, which are lower than 3%. The results are expressed as mmol Trolox equivalent/L. Vitamin C concentration was measured by FRASC method using ascorbate oxidase (20).

Measurement of total peroxide concentration

Total peroxide concentrations of plasma samples were determined by using FOX2' method with minor modifications (21,22). The FOX2 test system is based on oxidation of ferrous ion to ferric ion by various types of peroxides contained within the plasma samples, to produce a colored ferric-xylenol orange complex which absorbance can be measured. The FOX2 reagent was prepared by dissolving ammonium ferrous sulphate (9.8 mg) in 250 mM H2SO4 (10 ml) to give a final concentration of 250 µM ferrous ion in acid. This solution was then added to 90 ml of HPLC-grade methanol containing 79.2 mg butylated hydroxytoluene (BHT). Finally, 7.6 mg xylenol orange was added with stirring to make the final working reagent (250 µM ammonium ferrous sulphate, 100 µM xylenol orange, 25 mM H2SO4, and 4 mM BHT in 90 % vol/vol methanol in a final volume of 100 ml). The blank working reagent contained all components of the previous reagent except only ferrous sulphate. Aliquots (200 µL) of plasma were mixed with 1800 µL FOX2 reagent. After incubation at room temperature for 30 min, the vials were centrifuged at 12 000 g for 10 min. Absorbance of the supernatant was then determined at 560 nm. Total peroxide content of plasma samples was determined as a function of the absorbance difference between test and blank tubes using a solution of H2O2 as standard. The coefficient of variation for individual plasma samples was less than 5%.

Oxidative stress index

Percent ratio of total peroxide level to TAC level was accepted as oxidative stress index (22,23). To perform the calculation, the result unit of TAC, mmol Trolox equivalent/L, was changed to µmol Trolox equivalent/L and the OSI value was calculated as below formula;

OSI = ((Total peroxide, µmol/L) / (TAC, µmol Trolox equivalent /L) X 100).

Mononuclear cell DNA damage determination by alkaline comet assay

Peripheral mononuclear cell isolation for the comet assay was performed using the Histopaque 1077 (Sigma). An amount of 1 ml heparinized blood was carefully layered over 1 ml Histopaque and centrifuged for 35 min at 500 X g at 25°C. The interface band containing lymphocyte were washed with phosphate buffered saline (PBS) and then collected by 15 min centrifugation at 400 X g. The resulting pellets were resuspended in PBS to obtain 20 000 cells in 10 µl. Membrane integrity was assessed by means of Trypan Blue exclusion method.

The Comet assay was performed according to Singh et al. (24), with the following modifications. Thus, 10 µl of fresh blood (around 20 000 cells) was mixed with 80 µl of 0.7% low-melting agarose in PBS at 37°C. Subsequently 80 ml of mixture was layered onto a slide pre-coated with thin layers of 1% normal melting point agarose (NMA), and immediately covered with a coverslip. Slides were left for 5 min at 4°C to allow the agarose to solidify. After removing the coverslips, the slides were submersed in freshly prepared cold (4°C) lysing solution (2.5 M NaCl, 100 mM EDTA-2Na, 10 mM Tris-HCl, pH 10 - 10.5, 1% Triton X-100 and 10% DMSO added just before use) for at least 1 h. Slides were than immersed in freshly prepared alkaline electrophoresis buffer (0.3 mol/L NaOH, and 1 mmol/L Na_2EDTA, pH>13) at 4°C for unwinding (40 min) and then electrophoresed (25V/300 mA, 25 min). All the steps were carried out under minimal illumination. After electrophoresis, the slides were stained with ethidium bromide (2 µg/ml in distilled H2O; 70 µL/slide), covered with a coverslip and analyzed using a fluorescence microscope (Nikon). Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analyzed visually from each subject. Each image was classified according to the intensity of the fluorescence in the comet tail and was given a value of either 0, 1, 2, 3 or 4 (form undamaged class 0 to maximally damaged class 4), so that the total score of slide could be between 0 and 400 arbitrary units (AU).

Statistical Analysis

Values are expressed as a mean ± standard deviation or percentage. Comparisons of the results were made by using paired t-test. Bivariate analysis of the associations between each risk factor and oxidative/antioxidative parameters was performed with Pearson's correlation coefficient. For multiple linear regression, factors showing a value p<0.05 in bivariate analysis were selected. Multiple linear regression analysis was used to determine the change of TAC, total peroxide, OSI, vitamin C and duration of TET and related factors. Statistical significance was considered at p<0.05. Data were analyzed with SPSS for Windows software.

Results

The demographic and clinical data are reported in Table 1. Antioxidative/oxidative characteristics and DNA damage of the subjects before and after the exercise are shown in Table 2. As seen in the Table 2, plasma TAC and vitamin C levels decreased, and total peroxide, and OSI levels increased after TET (p<0.001). There was an increased level of DNA damage after TET than that before but it was not statistically significant (p>0.05).

Changes of TAC, total peroxide, vitamin C and OSI were not significantly different in subjects with positive exercise test and negative exercise test (p>0.05). The changes of TAC and vitamin C levels more significant in male subjects than in females (p<0.05). There was not a significant difference in DNA damage change between subjects with positive and negative exercise test results (p>0.05). As it is seen in the Table 3, duration of TET was found to be significantly lower in female gender and in subjects with positive exercise test (p<0.05, respectively for both).

The bivariate correlation between change of oxidative/antioxidative parameters, age, BMI and waist circumference are given in Table 4. As seen in Table 4, significant negative correlations were found between changes of TAC and duration of TET and, changes of vitamin C and duration of TET (r=-0.416, p=0.002 and r=-0.234, p=0.014). There were no significant correlations between changes of OSI and others factors and, changes of DNA damage and age, BMI, waist circumference and duration of TET.

The correlations between the duration of TET and other parameters are listed in Table 5. Significant correlations were detected between duration of TET and age, change of TAC and change of vitamin C values (r=-0.229, p<0.015; r=-0.230, p=0.014;

Table 1. Clinical characteristics of the 113 participants of the study

Parameters	Mean ± SD
Age, years	46 ± 11
Male/ Female, n	69/44
BMI, kg/m^2	24 ± 5
Waist circumference, cm	89 ± 23
TET duration, min	7.7 ± 3.5

BMI- body mass index, TET- treadmill exercise test

Table 2. Oxidative/antioxidative parameters and DNA damage levels of the individuals' before and after exercise test

Parameters	Before TET	After TET	p
TAC, mmol Trolox Equiv./L,	1.78±0.86	1.72±0.75	<0.001
Vitamin C, µmol/L	98.0 ± 24.2	95.0 ± 33.4	<0.001
Total peroxide, µmol H2O2/L	12 ± 4	14 ± 3	<0.001
OSI, AU	0.72 ± 0.28	0.81 ± 0.22	<0.001
DNA damage, AU	163 ± 45	176 ± 51	0.095

Values are mean ± SD for variables
AU- arbitrary unit, OSI- oxidative stress index, TAC- total antioxidant capacity, TET- treadmill exercise test

r=-0.344, p<0.001; respectively). In multiple linear regression analysis (R square=0.384, p<0.001), change of vitamin C was independent predictor of TET duration (ß=-0.209, p =0.030).

Discussion

In this study, we investigated whether there is any alteration in TAC, total peroxide, OSI, vitamin C and DNA damage in subjects before and after TET. We found decreased TAC and vitamin C, and increased OSI and total peroxide levels after TET. In addition, we used comet assay to measure the level of DNA damage in freshly isolated individual peripheral blood mononuclear cell fractions before and after TET. The DNA damage score was not significantly higher after TET in any subject.

Free radicals and oxidants such as superoxide radical anion, hydroxyl radical and hydrogen peroxide are produced in metabolic and physiological processes (6). Oxidative effects of free radicals are controlled by exogenous antioxidants such as vitamins E and C, and also by endogenous antioxidants such as scavenger enzymes; superoxide dismutase, glutathione peroxidase and catalase, and albumin, bilirubin and uric acid. Under some conditions, increases in oxidants and decreases in antioxidants cannot be prevented, and oxidative/antioxidative balance shifts towards the oxidative stress (6).

Table 3. Oxidative/antioxidative parameters and DNA damage level, duration of treadmill exercise test, and age and genders of subjects

	cTAC, mmol Trolox Equiv./L	cVit. C, umol/L	cTP, umol H2O2/L	cOSI, AU	cDNA damage, AU	dTET, min
Treadmill stress test						
Positive, (n=31)	-0.69±0.08	-2.7±3.0	1.04±2.70	0.09±0.15	0.6±3.0	7.4±2.2
Negative, (n=82)	-0.68±0.08§	-3.9±4.0§	1.38±2.40§	0.10±0.15§	0.3±30§	8.0±1.7§
Gender						
Male, (n=81)	-0.08±0.08	-1.7±2.6	1.01±2.70	0.09±0.16	1.0±3.6	8.3±1.9
Female, (n=32)	-0.04±0.05‡	-3.60±2.05†	1.45±2.20§	0.10±0.13§	1.6±3.6§	6.0±1.6*

*, p<0.001; †, p<0.01; ‡, p<0.05, §; p>0.05
P- student's t test, treadmill stress test positive versus treadmill stress test negative, and male versus female
Values are mean ± SD
AU- airbitrary unit, cDNA dam.- change of DNA damage, cOSI- change of oxidative stress index, cTAC- change of total antioxidant capacity, cTP- change of total peroxide, cVit.C- change of vitamin C, dTET- duration of treadmill exercise test

Table 4. Bivariate analyses of oxidative, antioxidative parameters, duration of exercise test and risk factors of subjects

	cTAC		cVit C		cTP		cOSI	
Risk factors	r	p	r	p	r	p	r	p
Age	-0.157	0.098	-0.172	0.069	-0.086	0.363	-0.089	0.350
BMI	-0.061	0.519	-0.070	0.463	0.151	0.110	0.139	0.142
WC	-0.051	0.623	-0.090	0.363	0.141	0.193	0.129	0.152
Duration of TET	-0.230	0.014	-0.344	<0.0001	0.041	0.667	0.063	0.505

BMI- body mass index, cOSI- change of oxidative stress index, cTAC- change of total antioxidant capacity, cTP- change of total peroxide, cVit.C- change of vitamin C, WC- waist circumference

Table 5. Bivariate and multiple linear regression analyses for duration of exercise test and oxidative/antioxidative, DNA damage and other risk factors

	Bivariate analysis		Multivariate analysis	
	r	p	ß	p
Age	-0.229	0.015	-0.094	0.247
Waist circumference	0.148	0.076		
Body mass index	0.078	0.467		
cTAC	-0.230	0.014	-0.136	0.160
cVit.C	-0.344	<0.001	-0.209	0.030
cTP	0.041	0.667		
cOSI	0.064	0.503		
cDNA damage	0.130	0.170		

cOSI- change of oxidative stress index, Ctac- change of total antioxidant capacity, Ctp- change of total peroxide, cVit.C- change of vitamin C

Measuring the free radicals is difficult because of its short life-span. The majority of studies investigating the effects of exercise on oxidative stress have focused on markers of free radical induced tissue damage (11). Exercise appears to increase free radicals and ROS, and these interact with lipids, DNA and proteins. These interactions degrade proteins and damage DNA-strand breakage and other genomic structures (25). It is well known that different exercise protocols, training status, age and gender could play a role in oxidative/antioxidative parameters and DNA damage (26-28). We have chosen TET, which is a standard form of exercise. Some parameters of oxidative stress may not change after exercise, and may reach their maximal levels only hours or even days after the end of exercise (28-30). Some investigators have failed to observe any signs of exercise-induced oxidative stress immediately after exercise (27, 31, 32). Although some studies suggest that exercise training enhances antioxidant capacity, the causal mechanisms are not clearly known yet (31,33). Studies have used different markers of antioxidant status and different training levels of subjects. In this study, increased OSI and total peroxide levels, decreased TAC were observed at immediately after TET. To the best of our knowledge, this is the first study, which has examined the acute effect of TET on oxidative/antioxidative parameters and DNA damage after TET in untrained voluntary subjects with suspected coronary artery disease.

Some studies have observed exercise-induced DNA damage (27,31,34) but others have failed to detect such damage (35,36). Results from human studies showing the effects of exercise on DNA damage are depend on duration and degree of exercise, and training status (27,29). Acute or prolonged moderate exercises have not produced DNA damage, but long-period and intense exercises cause an increase in DNA damage (37).

Previous studies found that TAC levels were increased and some antioxidants were reduced immediately after an exercise (38-40). Some studies have shown decrease in glutathione and increase in glutathione peroxidase activity after exercise, which return to baseline levels by 1 h post exercise (41,42). Camus et al (43) have taken blood 20 min from the beginning of exercise, immediately after exercise and 20 min after exercise, and examined plasma ascorbic acid concentrations. Plasma ascorbate concentration decreased 20 min after beginning of the run, and it also continued after exercise, and approached resting levels at 20 min after exercise. In this study, we showed that vitamin C was significantly decreased after TET. It is not clear why studies examining concentrations of vitamin C during and 1 h after exercise shows various responses. This variability may be due to the differences in the used mode of exercise, the time points examined, the level of training of the subjects, environmental factors (e.g., altitude).

It is widely assumed that oxidative stress is detrimental to exercise performance, but there is little experimental evidence to support this. Although antioxidant supplementation has been shown to decrease exercise-induced oxidative stress in humans (11,44,45), there is no convincing experimental evidence that this is accompanied by an increase in exercise performance in healthy human subjects (46-49). We observed that vitamin C is a predictive factor for duration of TET . One limitation of the study is that diet during the training period was not controlled. In fact, study requires that a participant follow the same diet with in seven days preceding each blood sampling. The lack of control training group and calculation of VO2 max were other limitations of this study.

Conclusions

Our findings indicate that acute effects of TET are manifested by increase in oxidants and decrease in total antioxidant capacity which lead to oxidative stress, though DNA is not affected. Supplementation of vitamin C may increase the duration of TET. Further randomized clinical studies are needed to explain this status.

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