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Assessment of DNA Damage and Protective Role of Vitamin E Supplements after Exhaustive Exercise by Comet Assay in Athletes

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Abstract

Exhaustive physical exercise is associated with an increased oxidative activity and antioxidants are widely used as supplements, to prevent the deleterious effects of endogenous reactive oxygen species. The purpose of this study is to investigate exercise induced oxidative DNA damage and the possible protective effect of vitamin E supplementation. Comet assay was carried out in 23 athletes who were competitive rowers and physical education students of a Sports school. The blood samples were tested at baseline, 24h after performing maximal exercise and all subjects took antioxidant supplementation (vit E 400 IU/day) for 60 days and the above tests were repeated. The mean percentage of DNA damage in tail (% DNA_T) in rowers, were higher than in physical education students, (8.32±0.51, 5.99±0.52, respectively). A significant decrease in the extent of %DNA_T was observed in athletes who used vitamin E supplementation for 60 days compared to their initial DNA damage evaluations.

Keywords: DNA damage; Exhaustive physical exercise; Vitamin E supplementation; Comet assay

Introduction

Besides the many known health benefits of physical activity, exercise enhances the generation of free radicals and reactive oxygen species (ROS) by an increase of oxygen uptake, leading to oxidative stress which can result in damage in all cellular macromolecules such as DNA, lipids, and proteins [1-3] and are expected to be more prominent in high intensity exercise [4-6]. It is also known that acute or prolonged moderate exercises have not produced DNA damage, but long-period and intense exercises can cause an increase in DNA damage [7-11].

A number of publications have shown that, antioxidants protect the integrity of DNA from the damaging potential of genotoxicants and are capable of eliminating ROS generated oxidative stress [12-15]. The possible preventive effect of antioxidants in exhaustive exercise induced DNA damage has also been shown [16,17]. Especially, vitamin E, the major lipid-soluble antioxidant, has been discussed as a potent peroxyl radical scavenger and contributes to the antioxidant defense system [16,18-20]. Recently, the comet assay is being used with increasing popularity in human biomonitoring studies and the assay is an ideal biomarker due to its sensitivity and reliability in detecting DNA damage and repair [21-23]. Therefore, the purpose of this study was to investigate the possible DNA damaging effect of exhaustive physical exercise in students at the School of Physical Education and Sports in Turkey and to examine whether vitamin E (400 IU/day) supplementation for 60 days has an effect in oxidative damage using the comet assay.

Material and Methods

Study participants and design

The study participants were male competitive rowers (n=12) and students of physical education (n=11) at the School of Physical Education and Sports of Marmara University. The competitive rowers participated in recreational sport activities more than 3-3.5 hour/day, 10 times/week. The physical education (PE) students were moderately physically active and participated in sport activities more than 1 hour/day, 2 times/week.

The rowers performed the three staged exercise test on the Concept II DR rowing ergo meter. The first stage consisted of a graded exercise test (GXT) starting from 100 W till reaching peak power with increments of 40 W/min. In the second and third stages the rowers were instructed to complete a 2000m rowing session with steady state power output at 80% of peak power. The PE students performed the exercise test on the Ergo line 900R bicycle ergo meter. After completing the GXT (40 W/min increments), the PE students cycled constantly at 70% of peak power for 6 minutes. The stages were separated by 10 minutes of recovery. In both groups all subjects experienced severe fatigue toward the end of each stage. This was evidenced by their self report, observation and heart rate recordings.

The Institutional Review board of Marmara University approved the study protocol. Each participant was interviewed with a questionnaire which covered a detailed medical, lifestyle, smoking habit, dietary history including variables known to induce the comet frequency. Any subject who had an intake of supplement, alcohol and smoking habit were not included to the study. We also ensured that all the subjects had not been taking any medications nor had any kind of genetic illness in order to avoid confounding factors. All subjects were healthy and instructed to abstain from physical exercise and training

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48 hours prior to each test. Peripheral blood samples for the comet assay were obtained 24 hours before the start and 24 hours after their incremental exercise. All athletes received supplements of a daily dose of 400 IU vitamin E for 60 days and the assay was repeated.

Blood sampling

Peripheral blood was collected from volunteers in sterile disposable syringes, transferred into heparinized tubes. Unstimulated lymphocytes were isolated by histopaque 1077 density gradient centrifugation and washed in phosphate-buffered saline (PBS), then resuspended in ice-cold PBS at cells, respectively for identical handling of samples from all groups. The viability was tested by trypan blue exclusion. The number of dye-excluding cells was always greater than 90% in all analyzed samples.

Comet assay (Alkaline single cell gel electrophoresis technique)

The alkaline comet assay was performed using an adaptation of the method of Singh et al. [21]. Fully frosted microscope slides were dipped briefly into 0.7% hot (60°C) normal melting agarose (NMA) prepared in PBS. The slides should be dried overnight at room temperature and then stored at 4°C until usage. Cells were mixed with 0.7% low melting point agarose (LMA) and placed on microscope slides. The slides were maintained on an ice-cold flat tray for 15 minutes to solidify. Slides were then carefully immersed in cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10) with 1% Triton X-100 and 10% DMSO added just before use, for at least 1 hour at 4°C.

Electrophoresis and staining

The slides were removed from the lysing solution, drained and placed in a horizontal electrophoresis tank. The tank was filled with fresh electrophoresis buffer (0.3 M NaOH, 1mM EDTA, pH 13) to a level in order to cover the slides. Before electrophoresis, the slides were left in the solution for 20 minutes to allow the unwinding of the DNA and the expression of alkali-labile damage. Subsequently, the DNA was electrophoresed for 30 minutes at 300 mA and 15 V. To prevent additional DNA damage, all steps described above were conducted in the dark and at 4°C. After electrophoresis, the slides were taken from the tank and washed three times (5 minutes each) with 0.4 M Tris buffer, pH 7.5 to neutralize the excess alkali. Then 50 µl ethidium bromide (EtBr - 20 µl/ml) was added to each slide. The slides were covered with a cover-slip, stored in a humidified box at 4°C and analyzed using a fluorescence microscope within 3-4 hours.

Image analysis

100 cells per subject were analyzed at 40x magnification, under a fluorescent microscope (Olympus, BX51) equipped with an excitation filter of 546 nm and a barrier filter of 590 nm. Comets are formed upon the principle of releasing damaged DNA from the core of the nucleus during electrophoresis. The percentage of DNA in tail (% DNA_T) on 100 cells per sample (two duplicate sample slides, 50 randomly selected cells scored per slide) were scored using image analysis software. (BAB Bs200Pro Image Processing and Analysis System of BAB Muh., Turkey). The total comet DNA (DNA_C) is the sum of the intensities located in the comet area. Head DNA (DNA_H) is the sum of the intensities located in the head area. Tail DNA (DNA_T) is the difference between total comet DNA and head DNA ($DNA_T = DNA_C - DNA_H$). Percent tail DNA (% DNA_T) is calculated as $100 \times DNA_T / (DNA_T + DNA_H)$. These results were expressed as a mean percentage tail DNA (% DNA_T) ± SD [24]. The analysis was performed blindly by one slide reader.

Statistical analysis

A statistical comparison between the mean and standard deviation (SD) were calculated for each parameter. A statistical comparison between the mean %DNA_T within the competitive rowers and physical education students were analyzed using the non-parametric Mann-Whitney U-test. P value ≤ 0.05 was considered to indicate statistical significance. All samples were coded at the time of preparation and scoring. SPPS 11.0 statistical software (SPSS Inc, Chicago, IL, USA) program was used for statistical analysis.

Results

General information of competitive rowers and PE students with respect to age (years), weight (kg), height (cm), body mass index (kg/m^2) are demonstrated in Table 1. The difference in physical performance between athletes was inevitable due to their obvious difference in training and physical fitness status. This is reflected in the total exercise time which was longer for the competitive rowers, and also in their higher power output.

The mean %DNA_T in competitive rowers and PE students prior to exercise, 24 hours after exercise and after 2 months of vitamin E supplementation, expressed as Mean ± SD. are presented in Table 2. The pre-exercise values of mean %DNA_T were significantly higher in rowers (8.32 ± 0.51) compared with PE students (5.99 ± 0.52) ($p \leq 0.001$). The mean %DNA_T in rowers 24 hours after the exercise increased (15.31± 0.67) significantly as compared to their pre-exercise values (8.32 ± 0.51) ($p \leq 0.001$). However, the pre-exercise statistical comparison in PE students without Vit. E (5.99 ± 0.52) or after Vit. E supplementation (5.65 ± 0.47) were not significant ($p > 0.05$) although the conditions were same.

The pre-exercise values before and after vitamin E consumption in rowers were 8.32± 0.51 and 6.27 ± 0.47, respectively and showed that the pre-exercise %DNA_T values in rowers decreased significantly ($p \leq 0.001$) after 60 days of vitamin E consumption. Competitive rowers had a p-value significantly higher than the mean % DNA_T observed for PE students in every measured parameter as demonstrated in Table 2.

The photographs in Figure 1, demonstrates the 24 hour after an exhaustive exercise comet assay patterns of a competitive rower and a PE student measured before and after 2 months of Vit. E supplementation.

Subjects	Physical education students (n=11) Mean ± SD	Competitive rowers (n=12) Mean ± SD
Age (years)	21.80 ± 3.80	22.00 ± 3.80
Weight (kg)	71.60 ± 12.80	79.60 ± 6.20
Height (cm)	176.60 ± 6.50	183.8 ± 4.90
BMI (kg/m^2)	22.84 ± 3.12	23.54 ± 1.49

Table 1: General information of the physical education students and competitive rowers.

DNA damage in lymphocytes (%DNA_T)			
	Competitive Rowers n=12	**PE Students n=11**	***P* value**
Pre-exercise	**8.32 ± 0.51**	**5.99 ± 0.52**	<0.001
24 h After-exercise	**15.31 ± 0.67**	**12.95 ± 0.49**	< 0.001
Vit. E Supplementation			
Pre-exercise	**6.27 ± 0.47**	**5.65 ± 0.47**	< 0.001
24 h After-exercise	**13.90 ± 1.01**	**12.19 ± 0.53**	< 0.001

Table 2: The percentage of DNA in tail (%DNA_T) in lymphocytes of competitive rowers, and physical education (PE) students prior to exercise, 24 hours after exercise and after 2 months of vitamin E supplementation (400 IU/day), expressed as Mean ± SD.

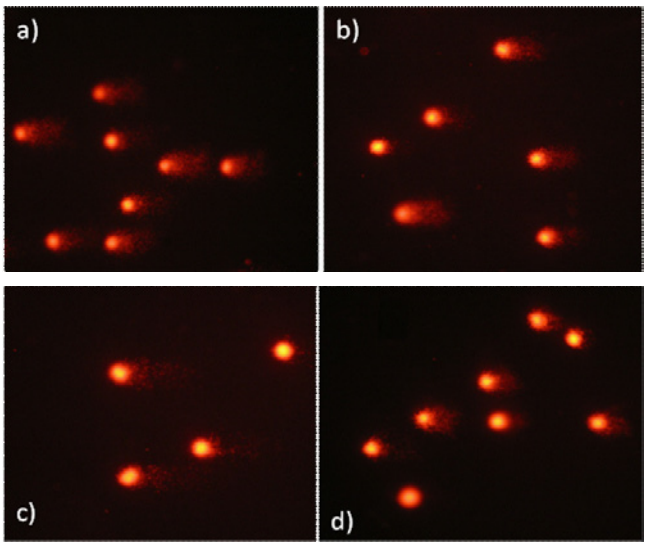

Figure 1: (a) Comet from a competitive rower measured 24h after an exhaustive exercise, (b) comet belonging to same subject after 2 months of vitamin E supplementation also measured 24h after an exhaustive exercise, (c) Comet from a PE student measured 24h after an exhaustive exercise (d) comet belonging to same PE student after 2 months of vitamin E supplementation also measured 24h after an exhaustive exercise.

Discussion

The results of the present study demonstrate an increase in percentage of cells with tails and an increase of mean DNA migration length in peripheral blood lymphocytes of physically active athletes. This situation is in accordance with the view of Sjödin et al. (1990) and Tsai et al. (2001) that energy demand during strenuous physical exercise causes an increase in oxygen uptake and supply to the active tissues, and a concomitant increase occurs in free radical production (ROS) which affects the capacity of endogenous cellular defence systems to neutralize these reactive species [25,26]. Produced ROS can permeate into cell nuclei and induce oxidative DNA damage and may also react with membranes (lipids), proteins, nucleic acids, and other components to initiate /cause cellular damage and degeneration. Tice et al. (1990) were the first to report effect of exercise by the comet assay in jogging persons [7]. A series of studies using the Comet assay from Günter Speit's laboratory have also addressed various aspects of the effect of exercise [27]. Our results, further support previous studies that report a positive relation between exercise and DNA damage by comet assay conducted on athletes doing various types of exercise. Niess et al. (1996) observed a clear and significant increase of DNA migration 24 hours after exercise in treadmill runners [28] and Mergener et al. (2009) measured the comet index in older adults that do exercises regularly such as walking and muscle building, and found damaged DNA levels greater than at baseline after physical activity [3]. Recently, Kim et al. (2010) have compared the DNA damage between weight trainers, runners and orbotron training group to demonstrate the effect of different types of exercise and training type on oxidative DNA damage and their results indicate that various parameters can differ depending on the type of physical exercise [29]. Their observation is also in accordance with the results of this study, because % DNA_T was more apparent in competitive rowers than PE students due to exercise type performed and training status. Moreover, oxidative DNA damage in lymphocytes using endonuclease III and increased formation of reactive species by phagocytes was found in runners during high-intensity marathon [30].

These observations that exhaustive physical exercise causes severe mutations have also been confirmed by various biomarkers such as the micronucleus (MN) test [31] or in urinary excretions such as acetone, propanal, pentanal, malondialdehyde (MDA) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) which was significantly correlated with training status of the volunteers [32].

Despite a strong effect in the comet assay, elevated frequencies of MN in lymphocytes and with a multi-parameter set of biomarkers with positive damage, there are number of other studies presenting a controversial picture about the situation. 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, exercise protocols, age and gender which may play a role in oxidative DNA damage.

In recent years, vitamin E has been utilized in various studies using the comet assay for its protective role from genotoxicants [13-15]. In accordance with these views, our aim was to determine the effects of vitamin E supplementation on exercise-induced oxidative stress, in other words to evaluate whether antioxidants are able to protect the integrity of the genetic material. Therefore all athletes continued their routine daily exercise and took vitamin E (400 IU/day) supplementation for two months. The mean %DNA_T in all athletes after the vitamin E (400 IU/day) supplementation decreased significantly compared to their initial %DNA_T measurements. However, the results of studies about exercise-induced DNA damage and antioxidant supplementation are diverse. Hartmann et al. (1995) applied multivitamin pills, vitamin E (3x800 mg or 1200 mg/daily) for 14 days prior to a run and intake of vitamin E 1200 mg clearly reduced the exercise induced DNA damage [16]. Supplementation with vitamin E 400 IU for periods of 3 weeks or with different doses of vitamin C (400 mg, 500- and 1000-mg) given for 2 weeks, or the combined treatment of vitamins C + E have yielded mixed findings in relation to suppressing exercise-induced oxidative stress. Collectively, the data are mixed in relation to antioxidant supplementation and exercise-induced oxidative stress, but indicate that vitamin E may act to suppress lipid peroxidation, whereas vitamin C alone does not appear to have this ability [33-36].

Mastaloudis et al. (2004) studied runners during ultramarathon by randomly assigning 1000 mg vitamin C or 400 IU *RRR-alfa-tocopherl acetate* and assessed the DNA damage with comet assay. No statistical differences between the treated men were found, but enhanced recovery in women was observed [17]. Bloomer et al. (2006) assigned aerobically trained men and women to one of the three treatments including; 400 IU of vitamin E, 1 g of vitamin C supplementation and to fruit and vegetable juice powder concentrate for 2 weeks to compare the biomarkers of oxidative stress before and after aerobic exercise and observed no impact on plasma MDA or 8-OHdG [37].

In conclusion, with all the discrepancies in above findings including our study, it would have been very useful to measure the effect of antioxidant supplementation and ROS activity in all subjects by using cell based assay kit (such as DCFH-DA) in parallel with the genotoxicity assays. However, our findings with the comet assay suggest that it is important to keep in mind that exhaustive exercise seems to increase the production of oxygen radicals in humans and on the other hand, vitamin E supplementation provides positive health benefit on the caused damage. Finally, exercise is regarded as promoting good health and well being, but excessive exercise is associated with oxidative stress reflected by higher levels of DNA damage. Therefore our results are important to inform and educate athletes who exercise heavily for their

eating attitudes and lifestyle, preferably be supervised by a dietician for optimal functioning.

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