Exercise-induced oxidative stress leads hemolysis in sedentary but not trained humans

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Free radicals are known to play a pivotal role in tissue damage, as well as have an adverse effect on erythrocytes (2, 36). It is well known that there is a close relationship between oxidative stress and exercise. In addition to the emergence of free radicals from mitochondrial leakage owing to enhanced oxygen consumption, ischemia-reperfusion process and leukocyte activation may also contribute to oxidative stress during exercise (19, 20), especially in extramusscular tissues (heart, liver, brain) and erythrocytes. It is well documented that exercise-induced oxidative stress occurring in various tissues and blood can be prevented by antioxidant interventions in animals and humans (19, 31). Furthermore, antioxidant vitamin supplementation also prevents certain hemolytic situations due to nonexertional oxidative stress (14, 18, 29, 40).

Our laboratory previously demonstrated that a single exhaustive exercise period in sedentary rats promoted oxidative stress and led to structural and functional deterioration in erythrocytes (32). One-month antioxidant vitamin treatment prevented exercise-induced oxidative stress and also erythrocyte deterioration in sedentary rats after exhaustive exercise. However, this impact was not demonstrated in exercise-trained rats. The significant differences between red blood cells in rats and humans prevent extrapolation of findings from one species to the other. There are several structural differences between rat and human erythrocytes, including cell size, membrane lipid and protein compositions, and membrane surface charge (4, 17). These structural differences between human and rat erythrocytes may lead to altered deformability, aggregation properties, and also hemolysis (3, 4, 28, 30).

The aim of the present study was to determine the contribution of oxidative stress to exercise-induced hemolysis in sedentary and trained men before and after antioxidant treatment with A, C, and E vitamins.

MATERIALS AND METHODS

Subjects, Protocols, and Sampling

Twenty young male students from the Medical School and School of Physical Education and Sports of Akdeniz University volunteered for the study. Subjects were nonsmokers and had no apparent health
problems. The subjects were informed about procedures involved and any discomfort associated with the experiment before giving their written consents. The study was approved by the Ethical Committee of Akdeniz University and Ministry of Health. One student of the School of Physical Education and Sports was withdrawn from the experiment by his own desire, and one student from the Medical School broke his arm during the experimental period. Nine students from the Medical Faculty were included in the sedentary group. Students in this group were not engaged in any regular exercise activity, either personally or as a group activity. Nine students from the School of Physical Education and Sports who were on the university handball team were included in the trained group. Maximal aerobic capacity of these subjects was assessed by a computerized, breath-by-breath analyzing system (Vmax Spectra 29 LV, Sensormedics). The subjects performed the Bruce protocol on a motorized treadmill ergometer. Maximal aerobic capacity corresponded to the plateau in oxygen consumption, despite the increment in the workload. The physical, hematological, and training characteristics of subjects are presented in Table 1.

All subjects participated twice in a cycling exercise test. After a 2-min warm-up, the workload test was started at 50 W and increased by 50 W every 2 min, while the subjects maintained a pedal frequency of 50 rpm. The tests lasted for 8–12 min, the time at which the subjects expressed their exhaustion or when maximal heart rate was attained, calculated as 220 – age. At the end of the workload test, all subjects had a 2-min cool-down. All experiments were conducted in an exercise laboratory, where room temperature varied between 20 and 23°C and humidity was 40 ± 10%. The first exercise test was performed before antioxidant vitamin treatment. Regular daily vitamin intake started 1 wk after the first exercise test and continued for 2 mo. All subjects utilized commercially available pills of vitamin A (β-carotene, 50 mg/day), vitamin C (ascorbic acid, 1,000 mg/day), and vitamin E (α-tocopherol, 800 mg/day). The exercise test was repeated after the treatment period in both groups. Both tests were applied to the trained subjects 48–72 h after their last training session. Regular physical training continued during antioxidant treatment.

Venous blood samples were obtained from an antecubital vein before and after the aerobic exercise test and anticoagulated with sodium heparin (15 IU/ml). The first sample (basal) was obtained 10 min before the start of the exercise protocol. The remaining blood samples were obtained at 2, 12, and 24 h after the exercise bout. These samples were used for the measurement of erythrocyte mechanics and hemolysis parameters, lipid peroxidation, protein oxidation, antioxidant defense system, and density separation of erythrocytes. Blood lactate concentrations were also measured 5 min after termination of exercise by a lactate analyzer using BM-Lactate test strips (Accusport, Boehringer Mannheim, Mannheim, Germany).

<table>
<thead>
<tr>
<th>Table 1. Physical, hematological, and training characteristics of subjects</th>
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<tr>
<td><strong>Sedentary Group</strong></td>
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<tr>
<td>Age, yr</td>
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<tr>
<td>Weight, kg</td>
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<tr>
<td>Height, cm</td>
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<tr>
<td>Erythrocytes count, ×10¹²/L</td>
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<tr>
<td>Hematocrit, %</td>
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<td>Hemoglobin, g/dl</td>
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<td>Training time, h/wk</td>
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<td>Resting pulse rate, beats/min</td>
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<td>V̇O₂max, ml/min·kg⁻¹</td>
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Values are means ± SE. V̇O₂max, maximal aerobic capacity. *P < 0.05, †P < 0.01 difference from sedentary group.

Erythrocyte fragility and mechanical parameters

Osmotic fragility. Erythrocyte fragility was determined osmotically by the method of Beutler (8). Briefly, heparinized whole blood (10 μl) was added to tubes with increasing concentration of buffered salt solution (pH 7.4; 0, 0.3, 0.35, 0.4, 0.45, 0.5, 0.55, 0.6, 0.7, 0.9%). The tubes were gently mixed and incubated at room temperature for 30 min. Then the samples were centrifuged at 2,000 rpm for 10 min, and supernatants were collected. Optical density of the supernatant was measured by a spectrophotometer at 540 nm. Hemolysis in each tube was expressed as a percentage, taking as 100% the maximum value of absorbance of the distilled water (0% concentration). The half-maximal (50%) concentration values were calculated from the percentage of hemolysis in buffered salt solutions at various concentrations by GraphPad Prism 3.0 software program.

Erythrocyte deformability. Red blood cell deformability was measured at various fluid shear stresses by laser diffraction analysis using an ekactymeter (LORCA, RR Mechatronics, Hoorn, The Netherlands). The principle of the system was described elsewhere in detail (16). Briefly, a low-hematocrit red blood cell suspension in an isotonic solution of polyvinylpyrrolidone (6% in PBS, viscosity: 25.3 cP) is sheared in a Couette system composed of a glass cup and a precisely fitting bob, with a gap of 0.3 mm between the cylinders. A laser beam is directed through the sheared sample, and the diffraction pattern produced by the deformed cells is analyzed by a microcomputer. Based on the geometry of the elliptical diffraction pattern, an elongation index (EI) is calculated as follows: EI = (L – W)/(L + W), where L and W are the length and width of the diffraction pattern, respectively. EI values were determined for nine shear stresses between 0.5 and 50 Pa. The shear stress required for half-maximal deformation (S50) was calculated from the data set for each measurement by using a Lineweaver-Burk analysis procedure (5). Increased S50 values represent the decreased deformability of erythrocytes. Briefly, the shear stress-EI curve was linearized by plotting the reciprocal of EI vs. the reciprocal of shear stress, with the x-intercept of this line corresponding to the negative reciprocal value of S50. All measurements were performed at 37°C.

Plasma hemoglobin concentration. A modified method, based on cyanmethemoglobin, was used for determining plasma hemoglobin concentrations, and the values are expressed as grams per deciliter (6).

Plasma haptoglobin concentration. A commercial nephelometric kit (Dade Behring, Marburg, Germany) was used for analyzing plasma haptoglobin concentrations.

Oxidative and Antioxidant Status Parameters

Thiobarbituric acid-reactive substance. Lipid peroxidation of erythrocytes was estimated by the measurement of thiobarbituric acid-reactive substance (TBARS), as described by Stocks and Dornanday (35), using 1,1,3,3-tetraethoxypropane as a standard. TBARS levels were determined by measuring absorbance at 532 nm after reaction with thiobarbituric acid in erythrocyte extracts.

Carbonyl derivative. Reactive carbonyl derivative content as a marker of protein oxidation was measured using the method of Levine et al. (21). Because in our pilot study we observed that reactive carbonyl derivative content reached peak values earlier than TBARS, we decided to use a 12-h time point for protein oxidation analysis.

Antioxidant status. The activities of three antioxidant enzymes in erythrocytes and plasma levels of antioxidant vitamins were evaluated before and after antioxidant vitamin treatment. Catalase (CAT; EC 1.11.1.6), superoxide dismutase (SOD; EC 1.15.1.1), and glutathione peroxidase (GPx; EC 1.11.1.9) activities were assessed by using the methods of Aebi (1), Misra and Fridovich (25), and Paglia and Valentine (27), respectively. Vitamin A and C levels were analyzed by using the methods of McCormic (23). Levels of vitamin E were determined, as described by Desai (13).


Density Separation of Erythrocytes

Density separation was applied to determine the ratio of erythrocyte age. Erythrocytes were separated, according to their density, using discontinuous Iodixanol (Optiprep, Nycomed Pharma As, Norway) density gradients (41). Iodixanol aliquots with densities between 1.075, 1.085, 1.095, 1.105, and 1.115 g/ml (2 ml each) were layered on top of each other in a test tube. One milliliter of whole blood was carefully layered on top of the least dense layer, and the tube was centrifuged at 2,500 g for 25 min at 22°C. Each layer was carefully separated, and the density distribution of erythrocytes was estimated by determining the hemoglobin concentration in each layer. Because erythrocyte density increases as a function of age (12), old cells accumulated at the bottom (dense) layers of Iodixanol.

Statistical Analysis

The results are expressed as means ± SE. Three-way ANOVA was used for statistical analyses. Training effect, antioxidant supplementation, and acute exercise effect were chosen as independent parameters. Paired t-test was used for comparison of nonrepeated parameters within the groups. Student’s t-test was used to compare nonrepeated measurements between two groups (physical, hematological, and training characteristics). P < 0.05 was accepted as significant.

RESULTS

All subjects completed both exercise protocols without problems before and after antioxidant treatment. Although maximal pulse rates and lactate concentrations were not different between groups, exercise durations of trained subjects were significantly greater than those of sedentary subjects before and after antioxidant treatment (P < 0.01 and P < 0.05, respectively) (Table 2). None of these three parameters was altered by antioxidant treatment in both groups.

Responses of Sedentary Subjects

Osmotic fragility, deformability, plasma hemoglobin concentration, and haptoglobin levels of the sedentary group are shown in Fig. 1. There were significant deteriorations in osmotic fragility and deformability in sedentary erythrocytes 24 h postexercise compared with basal levels before antioxidant treatment (P < 0.05). Meanwhile plasma hemoglobin concentrations increased, and haptoglobin levels decreased 24 h postexercise compared with basal values (P < 0.05), as is typical of intravascular hemolysis. Erythrocyte deformability 2 h postexercise was also significantly increased compared with basal levels (P < 0.05). There were no significant differences in osmotic fragility, deformability, plasma hemoglobin concentration, and haptoglobin levels of the sedentary group after antioxidant treatment.

Table 2. Maximal heart rate, exercise duration, and lactate concentrations of subjects

<table>
<thead>
<tr>
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<th>Before Antioxidant Treatment</th>
<th>After Antioxidant Treatment</th>
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<tr>
<td></td>
<td>Sedentary</td>
<td>Trained</td>
</tr>
<tr>
<td>Maximal pulse rate, beats/min</td>
<td>189.7±3.4</td>
<td>186.2±3.3</td>
</tr>
<tr>
<td>Exercise duration, s</td>
<td>569.7±19.5</td>
<td>662.0±23.3†</td>
</tr>
<tr>
<td>Lactate concentration, mmol/l</td>
<td>9.4±0.7</td>
<td>10.2±1.2</td>
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Values are means ± SE. *P < 0.05, †P < 0.01 difference from sedentary group.

TBARS and carbonyl derivative levels of the sedentary group are presented in Fig. 2. Values of TBARS 24 h postexercise and protein carbonyl content 12 h postexercise were significantly increased before antioxidant treatment compared with their basal levels (P < 0.01). TBARS levels 2 h postexercise were also significantly higher than basal values (P < 0.01). Two months of antioxidant vitamin therapy completely prevented the postexercise increase in TBARS and carbonyl derivative levels.
Responses of Trained Subjects

Osmotic fragility, deformability, plasma hemoglobin concentration, and haptoglobin levels of the trained group are shown in Fig. 3. There were no significant differences after the first exercise test in osmotic fragility, deformability, plasma hemoglobin concentration, and haptoglobin levels of the trained group compared with their basal values before antioxidant treatment, except deformability of erythrocytes 2 h post-exercise was significantly increased after antioxidant treatment ($P < 0.01$).

Oxidative stress parameters of trained subjects were almost identical to those of sedentary ones (Fig. 4). Before antioxidant treatment, protein carbonyl content 12 h and TBARS levels 24 h after exercise were significantly higher than their basal levels ($P < 0.05$). Antioxidant treatment also attenuated the increase in TBARS and carbonyl derivative in trained subjects.

Although we observed significant time-dependent alterations within group analyses, oxidative stress parameters, erythrocyte fragility, and mechanical parameters were not statistically different between the sedentary and trained groups, according to multiple-way variance analysis results. However, variance analysis showed significant interactions between time-related alterations and antioxidant supplementation for oxidative parameters (TBARS, protein carbonyl content), suggesting that antioxidant treatment with vitamins A, C, and E suppresses oxidative stress in both groups.

Density Separation of Erythrocytes

The density distributions of erythrocytes in sedentary and trained groups are shown in Fig. 5. Erythrocytes in both groups were mostly accumulated in the layers with densities between 1.095 and 1.105 g/ml. In density layer 1.095 g/ml, where younger erythrocytes accumulate, sedentary and trained erythrocyte percentages were 30.3 ± 3.2 and 42.6 ± 5.6%, respectively ($P < 0.05$). However, in density layer 1.105 g/ml, sedentary and trained erythrocyte percentages were 48.6 ± 1.7 and 42.4 ± 3.7%, respectively ($P < 0.01$). In the first three layers, where younger erythrocytes were concentrated, the percentage of erythrocytes from the sedentary group was 32.5%, whereas in the trained group it was 45.5%.

Antioxidant Status

SOD, CAT, and GPx activities in erythrocytes and antioxidant vitamin levels in plasma are presented in Figs. 6 and 7. There were no differences in SOD values between both groups
and within the groups before or after antioxidant treatment. CAT levels were significantly higher in trained subjects compared with sedentary subjects before antioxidant treatment, but not after treatment, whereas GPx levels were significantly increased in both sedentary and trained groups after antioxidant treatment ($P < 0.05$ and $P < 0.01$, respectively). There were no significant differences in GPx levels between the two groups. The measured antioxidant vitamin levels of sedentary and trained subjects were similar before antioxidant treatment. Plasma vitamin A, C, and E levels significantly increased in both groups after antioxidant treatment compared with their basal values.

**DISCUSSION**

The results of this study demonstrated that a single bout of rigorous exercise was sufficient to elevate oxidative stress markers and induce deterioration of erythrocyte structure and function in sedentary young men, which was preventable by 2 mo of antioxidant vitamin treatment. However, this impact has not been demonstrated in trained subjects. This study also showed that the difference between sedentary and trained erythrocytes may partly be dependent on the younger erythrocyte population present in trained subjects. It is well known that regular physical activity triggers some accommodative processes; thus the results of our study will be discussed separately on the basis of responses obtained from exercise trained and sedentary men.

**Responses of Sedentary Subjects**

Physical activity was demonstrated to lead to lipid and protein oxidation via oxidative stress in many studies (20, 31). Sedentary subjects in our study also exhibited elevated levels of TBARS and carbonyl derivative, considered as markers for lipid and protein peroxidation, respectively. Although most studies indicate that exercise-induced oxidative stress predominates in the period just after the exercise, lipid peroxidation may increase later after exercise (20, 22, 24, 43). This delayed increase in exercise-induced oxidative stress depends on leukocyte and macrophage activation and activation of xanthine

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**Fig. 4.** Oxidative stress parameters of trained subjects. A: TBARS; B: carbonyl derivative. Values are means ± SE. *$P < 0.05$, difference from corresponding basal value (before or after treatment).

**Fig. 5.** Percentage of erythrocytes distributed in density fractions of sedentary and trained subjects. Values are means ± SE. *$P < 0.05$, **$P < 0.01$: difference from corresponding sedentary value.

**Fig. 6.** Levels of antioxidant enzymes of sedentary and trained subjects. A: superoxide dismutase (SOD); B: catalase (CAT); C: glutathione peroxidase (GPx). Values are means ± SE. *$P < 0.05$, difference from sedentary group. †$P < 0.05$, ††$P < 0.01$: difference from the value before vitamin treatment.
oxidase due to the ischemia-reperfusion process rather than the emergence of free radicals from mitochondrial leakage due to enhanced oxygen consumption during exercise (20, 39, 44). As we demonstrated in our previous pilot study that the time course of postexercise induction of plasma protein carbonyl content is earlier than that observed with TBARS, we chose to present the protein carbonyl content results at 12 h instead of 24 h postexercise in this study. Davies and Goldberg (10, 11) concluded that lipid peroxidation and protein oxidation involve different mechanisms in vivo, and protein oxidation occurs more quickly than lipid peroxidation.

Exercise-induced oxidative stress occurring in various tissues and blood of experimental animals and human beings can be prevented by antioxidant interventions (19, 31). Taking advantage of these observations, we used a cocktail consisting of vitamins A, C, and E to enhance antioxidant defense. Plasma A, C, and E vitamin levels significantly increased after the 2-mo treatment period. The basal levels of oxidative stress markers, determined after the 2-mo treatment, were slightly higher, although not significantly, compared with those before the vitamin treatment. However, no change in TBARS and protein carbonyl content was observed after the vitamin treatment in the postexercise period. It was observed that oral antioxidant treatment for 2 mo significantly prevented exercise-induced oxidative stress.

Increased hemolysis caused by oxidative stress has been reported in several situations, such as sickle cell anemia, β-thalassemia, vitamin E depletion, hyperglycemia, and exposure to H2O2, halothane, and sulphur dioxide (7, 14, 18, 40). In certain conditions, hemolysis of erythrocytes due to nonexertional oxidative stress was prevented with antioxidant vitamin supplementation, and hemolytic resistance of erythrocytes was enhanced (7, 14, 18, 29). In our laboratory’s previous study (32), the prevention of exercise-induced oxidative stress by antioxidant vitamins was associated with reduced intravascular hemolysis due to exhaustive exercise in sedentary rats. We have now reported a decrease in oxidative stress and an alteration in the response of erythrocytes to exhaustive exercise in sedentary men who received antioxidant treatment for 2 mo.

Increased plasma hemoglobin concentration and decreased haptoglobulin levels, indicative of erythrocyte destruction, were detected in samples obtained from sedentary subjects 24 h after exhaustive exercise. Intravascular hemolysis was reported in several experiments as a consequence of exercise (32, 37, 42). The presence of intravascular hemolysis 24 h after exercise suggests the maintenance of some destructive effects on erythrocytes. This might be based on increased osmotic fragility and reduced erythrocyte deformability. Our laboratory has previously observed a similar deterioration in erythrocytes after swimming or treadmill exercise protocols (32, 43). All observed results indicate that antioxidant vitamin treatment prevented the deleterious effects of exhaustive exercise on erythrocytes by decreasing exercise-induced oxidative stress.

Many studies have revealed that erythrocyte deformability is impaired early in the postexercise period. Plasma volume alterations, increases in blood lactate level, and alterations in cation concentrations of erythrocyte are major factors responsible for deteriorations of deformability after exercise (9). We found erythrocyte deformability to be impaired (i.e., higher SS1/2) in both sedentary and trained groups 2 h postexercise. This increase was significant before vitamin treatment in the sedentary group and after vitamin treatment in the trained group. In addition, oxidative stress is also a factor that affects erythrocyte deformability during later periods after exercise in our study. Prevention of exercise induced oxidative stress associated with decreased rates of erythrocyte destruction attained by antioxidant treatment demonstrates the validity of this mechanism in sedentary men undergoing physical activity.

Fig. 7. Levels of antioxidant vitamins of sedentary and trained subjects. A: vitamin A; B: vitamin C; C: vitamin E. Values are means ± SE. †P < 0.05, ††P < 0.01: difference from the value before vitamin treatment.

Responses of Trained Subjects

Erythrocytes of trained subjects exhibited different responses to exhaustive cycling exercise compared with sedentary individuals before antioxidant treatment. Erythrocyte osmotic fragility and deformability values did not differ from basal 24 h after the exercise session in the trained group. Similarly, intravascular hemolysis markers, plasma hemoglobin, and haptoglobulin concentrations were not significantly changed after rigorous exercise. However, cycling exercise caused an increase in oxidative stress markers (TBARS and protein carbonyl content) before antioxidant therapy. The pres-
ence of erythrocyte deterioration in sedentary subjects during the postexercise period, but not in trained subjects, despite oxidative stress, indicates a significant difference in erythrocyte properties between the two groups. As expected, the increase in TBARS and protein carbonyl content in trained subjects was also prevented by antioxidant treatment. However, antioxidant treatment did not alter the indexes of erythrocyte mechanics and fragility.

Regular physical activity is known to enhance antioxidant enzymes’ activity primarily in striated muscle, heart, and also in erythrocytes (19, 20, 26, 31). Only the CAT levels in our trained subjects were significantly higher than in sedentary subjects before the treatment period, while the other enzymes measured did not exhibit significantly higher levels during both pre- and posttreatment periods. Nevertheless, higher antioxidant enzyme levels could not provide an explanation for different responses of erythrocytes to the exercise test, as oxidative stress was apparent in both groups before antioxidant treatment. On the other hand, we did not examine low molecular weight biological compounds (such as glutathione, uric acid, bilirubin) that have antioxidant function (19) and may play a role in the observed discrepancies between the two groups. Erythrocytes from the trained group are more resistant to oxidative stress, thus displaying an altered response.

The life expectancy of erythrocytes might also be another explanation for more resistant erythrocytes in our trained group. Trained people have a younger erythrocyte population than sedentary people because regular physical activity shortens the life span of erythrocytes (34, 37, 42). We performed density separation of erythrocytes as an approach to evaluate age distribution of red blood cells in our sedentary and trained groups. Erythrocytes become denser than younger cells and move to lower layers of density separation tubes. We determined that subjects in our trained group have significantly younger erythrocytes. By the reason that younger red blood cells are more resistant to oxidative stress (12), the response to exercise in trained individuals was different from that in the sedentary group. In addition to their resistance to oxidative stress, younger erythrocytes have higher 2,3-diphosphoglycerate content and are more deformable, which may contribute to increased athletic performance (34). Therefore, oxidative stress occurring during exercise might be accepted as an adaptive mechanism, which supports the increased population of younger erythrocytes indirectly obtained by increased destruction of older erythrocytes.

In conclusion, exercise affects erythrocyte properties and leads to hemolysis by an oxidative-mediated mechanism in sedentary, but not in exercise-trained, young men. Oxidative stress might be an important factor responsible for destruction of erythrocytes observed after acute, single-bout rigorous training in sedentary subjects and/or at the beginning of the training period.

GRANTS

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REFERENCES


