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ORIGINAL CONTRIBUTION

# Vitamin E supplementation does not increase the vitamin C radical concentration at rest and after exhaustive exercise in healthy male subjects

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**Summary** *Background* Extensive exercise may promote the formation of reactive oxygen species and subsequently contribute to tissue damage. A compound which can protect cells and tissues is vitamin E. The vitamin E radical, formed during the radical scavenging process, can be reduced by vitamin C resulting in a higher level of the vitamin C radical (semidehydroascorbate free radical). An increase of the vitamin C radical, however, is assumed to exert prooxidative effects. *Aim of the study* To elucidate whether supplementation of vitamin E and exhaustive exercise lead to an increase of the vitamin C radical in human plasma. *Methods* A placebo controlled, cross over study with 13 male volunteers was carried out. After an 8 day supplementation period with 500 I. U. D-α-tocopherol, the subjects performed two exhaustive treadmill runs. Blood samples were collected at rest, 0, 0.25, 1, 3, 24 and 48 h after exercise. Serum was separated and concentrations of D-α-tocopherol and ascorbic acid were determined by HPLC. Vitamin C radical levels in plasma were assessed by electron paramagnetic resonance (EPR). *Results* Vitamin E and C both showed a tendency to decrease between 3 h and 24 h after exercise. Vitamin C radical level remained stable during the whole period. Neither vitamin E supplementation nor exercise had any influence on the plasma concentration of the vitamin C radical. *Conclusions* Vitamin E supplementation under conditions of mild oxidative stress does not result in an increased vitamin C radical concentration.

**Key words** vitamin C radical · vitamin E · vitamin C · exercise · oxidative stress

## Introduction

Reactive oxygen species (ROS) are formed during exercise and are discussed to play an important role in exercise-induced tissue damage [1–5]. Evidence for an increased free radical generation due to exercise was provided by Davies et al. [6] and Jackson et al. [7] in rat muscle and more recently by Ashton et al. [8] in human serum after exhaustive exercise. To protect cells and tissues from ROS, enzymatic (Superoxide dismutase, glutathione peroxidase, catalase CAT, etc.) and non-enzymatic antioxidants (vitamins C, E, glutathione GSH and other thiols) exist. During vitamin E deficiency forma-

tion of ROS and mitochondrial dysfunction increases and endurance performance decreases in exhaustively exercised rats [6, 9–10]. Indeed, it was repeatedly documented that supplementation of vitamin E increases tissue resistance to exercise-induced lipid peroxidation [11–13]. Vitamin E protects cells from oxidative damage, which might occur during exercise as a result of increased free radical formation. In contrast to the protective effect of vitamin E against lipid peroxidation, it is argued that high doses of vitamin E might even exert pro-oxidative effects in human low-density lipoproteins [14]. An increased concentration of D-α-tocopherol in cellular membranes and increased ROS-formation during exercise might result in an increased concentration of vitamin C radicals. Ascorbic acid protects membrane and cytosolic components from oxidative stress. In erythrocyte membranes ascorbic acid reduces the tocopheroxyl radical to tocopherol [15]. The vitamin C radical may act as both a pro- or anti-oxidant in biological systems depending upon the physiological conditions [16]. To elucidate whether vitamin E supplementation and exhaustive exercise results in increased vitamin C radical concentrations we supplemented human volunteers with vitamin E and determined vitamin E and vitamin C radical levels before and after exercise.

## Methods

### Subjects

Thirteen male subjects, age: (26.5 ± 0.9) years, height: (180.7 ± 1.3) cm, weight: (74.1 ± 3.0) kg, corresponding to a body mass index BMI of 22.6 ± 1.1, were enrolled in the study. They did not perform any kind of specific sports and devoted less than 3 h per week to recreational and other physical activities. The subjects were non-smokers with normal dietary habits and did not take any medication or vitamin supplements. Further recommendations were given to avoid dietary imbalance with respect to vitamins E, C and β-carotene and subjects were advised to record food intake during the study period by using a standardized protocol (EBIS, Stuttgart, Germany). The study was approved by our institute's Ethics Committee and is in accordance with the recommendations in the Declaration of Helsinki (1996) for research on human subjects. All subjects gave informed consent to participate in the investigation.

We used a placebo-controlled cross-over design with a wash-out period of 28 days between parts A and B. Supplementation started on day 0 and lasted 8 days. The vitamin E or placebo capsules were taken each morning after breakfast with a minimum of 10 g fat (butter). On day 8 the subjects performed two exhaustive treadmill runs. In part A, the subjects randomly received 500 I. U. D-α-tocopherol (RRR-α-tocopherol, Optovit® fortissimum 500 capsules, Hermes Arzneimittel GMBH, Munich, Germany) per day (7 subjects) or placebo (6 subjects). In part B, the same subjects performed the intake of verum or placebo in a reversed manner.

### Exercise procedure

The exercise protocol started with a graded exercise test (incremental exercise test, IET) on the treadmill (Saturn, HP COSMOS, Traunstein, Germany), which was performed until exhaustion. Initial speed was 6 km $h^{-1}$ with an increment of 2 km $h^{-1}$ every 3 min. The incline of the treadmill was kept constant at 1 %. Capillary blood for lactate measurement was obtained from the earlobe after every stage. The running speed at the individual anaerobic threshold (IAT) was assessed according to Dickhuth et al. [17]. After a resting period of 15 min the subjects performed a continuous run (CR) on the treadmill until exhaustion at a running velocity of 110 % of the IAT. In exercise procedure of part B the subjects were advised to attain the identical running times in IET and CR as performed in part A which could be achieved by 11 of the 13 volunteers. Also, running speed during CR was the same in part A and B. Total running time (IET + CR) was 28.5 ± 0.8 min (D-α-tocopherol) and 28.6 ± 0.9 min (placebo), respectively (Table 1).

### Blood sampling

Venous blood samples were taken in a sitting position using EDTA as an anticoagulant at rest, 0, 0.25, 1, 3, 24 and 48 h after the end of CR. Whole blood aliquots for complete blood cell counts were kept at room temperature and the procedures of analysis were started within 1 h after collection. Whole blood (30 mL) was centrifuged (4 °C, 2200 g, 10 min) immediately after sampling and plasma aliquots were stored at –70 °C until further analysis within 2 weeks. A total of 5 mL of plasma for determination of ascorbic acid was treated with meta-phosphoric acid (1 mol $L^{-1}$ and 400 µL plasma; 600 µL meta-phosphoric acid) and also stored at –70 °C. Capillary blood for lactate measurements was obtained from the earlobe before, 0 and 5 min after the runs and after every stage for the determination of the individual anaerobic thresholds.

### Chemicals and solutions

Meta-phosphoric acid, ortho-phosphoric acid, butylated hydroxytoluene and 2-thiobarbituric acid were of highest grade commercially available and were purchased from Sigma, USA or Merck, Germany.

### Analytical procedures

Complete blood cell counts including hemoglobin, hematocrit and differential leukocyte counts were performed by an automated Coulter Counter (Cell Dyn 3500, Abbott, Germany). Lactate concentrations of hemolyzed capillary blood were measured electrochemically using a lactate analyzer (EBIO, Eppendorf, Germany). Plasma creatine kinase acitivity (CK) was determined in the clinical laboratory routine (Hitachi 717, Boehringer, Ingelheim, Germany).

The measurements of thiobarbituric acid reactive substances (TBARS) in plasma were performed as recently described [18] using the difference in absorbance at wavelengths $\lambda = 535$ nm and $\lambda = 572$ nm (Unicam UV 2 spectrometer, UK). For assessment of cholesterol concentrations in plasma, we used a colorimetric test (Chol MPR 1, Boehringer Mannheim, Germany). Post-exercise values of plasma parameters were corrected for changes in plasma volume in response to exercise according to Dill and Costill [19].

### Determination of D-α-tocopherol and ascorbic acid concentrations in plasma

Plasma concentrations of D-α-tocopherol were analyzed using a modification of an HPLC method previously described [20]. After precipitation of proteins with 200 µL ethanol, 200 µL plasma were extracted twice with 500 µL hexane. Hexane phases were separated, dried under a continuous stream of nitrogen and redissolved in 200 µL hexane. D-α-tocopherol was separated on a 250 × 4 mm Merck LiChrosphere CN column using hexane as eluent. Detection procedures were performed by UV ($\lambda = 293$ nm, UV/VIS Detector 432, Kontron, Germany). Plasma concentrations of D-α-tocopherol were adjusted for plasma cholesterol levels.

Determination of ascorbic acid concentrations in meta-phosphoric acid-treated plasma samples was performed using a modified reversed phase HPLC system equipped with a PED 300 Pulsed Electrochemical Detector (Biometra, Germany) and a $C_{18}$-column Grom-Sil 120 Amino-2 PA operated at 1.2 mL $min^{-1}$. Upper phases of thawed plasma samples were vortexed with methanol/$H_2O$ (2/8/v/v). After removal of proteins by centrifugation (16 000 g, 22 °C, 5 min) 20 µL of the upper layer was injected into the HPLC system. As the mobile phase, acetonitrile containing 50 mmol $L^{-1}$ $KH_2PO_4$ was used.

### Measurements of vitamin C radical by electron paramagnetic resonance spectroscopy (EPR)

Electron paramagnetic resonance spectra were recorded as recently described [21]. Briefly, determination was carried out at 20 °C (293 K) using a Bruker ESP 300E spectrometer, equipped with a Bruker $TE_{102}$ (ER4102ST) cavity, operating at 9.6 GHz with a 100 kHz modulation frequency. A microwave power of 6.3 mW was employed. Plasma samples were thawn at 20 °C in the dark, and 60 µL was immediately transferred to a flat cell. The spectra were recorded within 15 min after thawing. No exogenous oxidation catalysts were added. The vitamin C radical concentration was determined by calibration of the signal intensity with 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl after double integration of the first derivative spectra. 10 000 arbitrary units of the signal amplitude correspond to 0.14 µmol $L^{-1}$ of the vitamin C radical. Due to the construction of the flat cell and a special holder as well as fixed instrumental conditions the precision of the method varied in a small range compared to the boundary of the interindividual differences. Instrumental settings were the following: 1 G modulation amplitude, $4 \cdot 10^5$ receiver gain, 82 ms conversion time, 20.5 ms time constant, 8 scans.

### Statistics

Statistical analyses and graphics were computed with the statistical software package JMP (SAS Institute Inc., Cary, USA) and KaleidaGraph (Synergy Software, Abelbeck, USA) for Macintosh computers. Data are expressed as means ± standard error (SEM). Differences between post-exercise and resting values were evaluated using the Student *t*-test for paired values. Intraindividual differences between measurements with D-α-tocopherol and placebo were calculated for every sampling time and tested for significance by the Student *t*-test for paired values. A value of $P < 0.05$ was regarded as significant.

## Results

### Sample handling

Sample handling and storage does affect the determination of the vitamin C radical. Repeated freezing and thawing however, has little if any effect on the measured radical concentration, i. e., the error is within the normal range at low radical concentrations due to low signal to noise ratios (about 5 %) for at least 2 week's storage time. A more critical parameter is the time after thawing at which the spectra are taken. Fig. 1 shows the time dependence of the EPR signal of blood samples after thawing. The maximum loss of intensity during the time until the samples were measured (15 min) is below 10 %. Also, blood samples have to be centrifuged immediately after sampling and subsequently they must be frozen within 5 minutes.

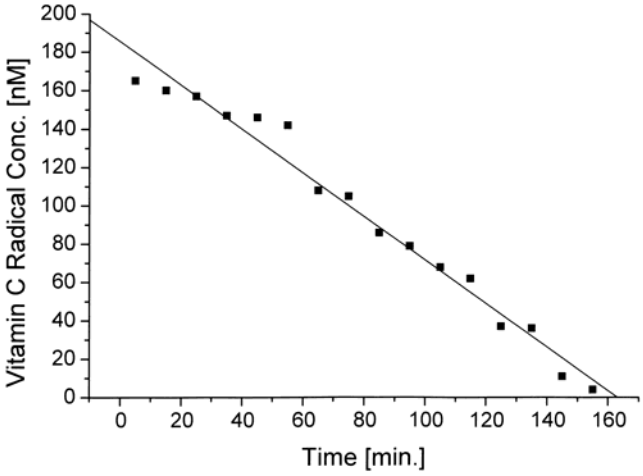


**Fig. 1** Time course of the decomposition of the vitamin C radical in human plasma samples at room temperature after thawing as determined by EPR spectroscopy

Exercise characteristics are given in Table 1. The data clearly show that the exhaustive exercise resulted in comparable blood lactate levels in the vitamin E treated and untreated group. D-α-Tocopherol supplementation induced a marked elevation of D-α-tocopherol in plasma which was still apparent 48 h after exercise (Fig. 2). Ascorbic acid did not exhibit exercise-related changes and was not affected by D-α-tocopherol supplementation (Fig. 2). Vitamin E and C both showed a tendency to decrease between 3 and 24 h after exercise. Vitamin C radical remained stable during the whole period. Neither vitamin E supplementation nor exercise had any influence on the plasma concentration of the vitamin C radical (Fig. 3). TBARS as the (tendency) marker for lipid peroxidation remained unchanged during the whole period in the treated and untreated group (Fig. 3).

Blood lactate values rose after IET and CR but did not show any differences between the verum and placebo group (Table 1). Plasma levels of CK peaked 24 h after exercise, but no effects of D-α-tocopherol on CK release from muscle were observed (Table 2).

## Discussion

### Effects of exercise

Exhaustive exercise as documented by lactate levels did not result in significantly increased lipid peroxidation (TBARS) nor an elevated vitamin C radical concentration, even in volunteers supplemented with high doses of vitamin E. Increases of the vitamin C radical result from enhanced ascorbate oxidation as a consequence of vitamin C-induced vitamin E radical reduction and this has been postulated to reflect oxidative stress [22]. In a

**Table 1** Exercise characteristics

| | D-α-tocopherol | Placebo |
|---|---|---|
| $V_{max}$ IET (km $h^{-1}$) | 16.0±0.4 | 16.1±0.4 |
| $LA_{max}$ IET ($mmol^{-1}$) | 10.7±0.9 | 10.2±0.8 |
| $T_{max}$ CR (km $h^{-1}$) | 10.5±1.0 | 10.6±1.0 |
| $LA_{max}$ CR (km $h^{-1}$) | 9.1±0.6 | 9.2±0.5 |
| Total running time (min) | 28.5±0.8 | 28.6±0.9 |
| IAT ($V_{IAT}$) (km $h^{-1}$) | 11.01±0.54 | 11.06±0.46 |

Values are means ± SEM, n = 13
*$V_{max}$ IET* maximal running velocity in the incremental exercise test
*$LA_{max}$ IET* maximal blood lactate in the incremental exercise test
*$T_{max}$ CR* running time in the continuous run
*$LA_{max}$ CR* maximal blood lactate after the continuous run

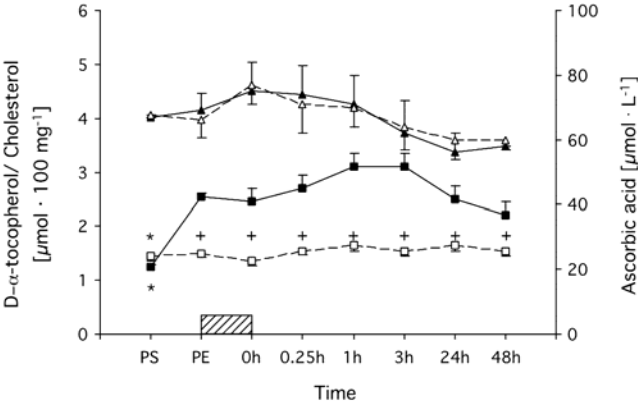


**Fig. 2** Cholesterol-adjusted D-α-tocopherol (■, □) and ascorbic acid concentrations (▲, △) in plasma before supplementation (PS), before exercise (PE) and at several time points after exercise and supplementation with D-α-tocopherol (straight line) or placebo (broken line), (mean ± SEM). * denotes significant changes compared to pre-exercise values ($P < 0.05$), + denotes significant differences between D-α-tocopherol and placebo ($P < 0.05$)

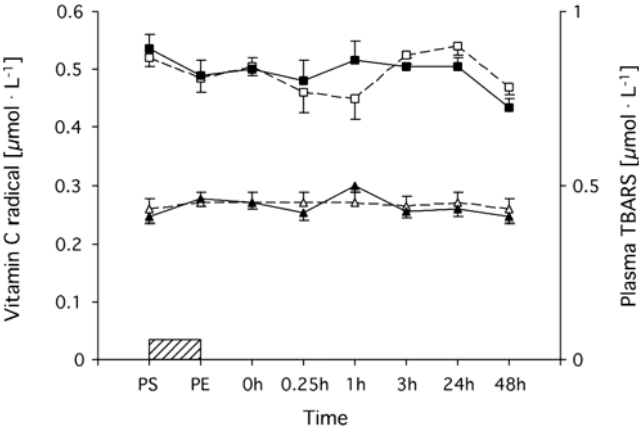


**Fig. 3** Vitamin C radical detected by EPR (■, □) and plasma TBARS concentrations as measured spectrophotometrically (▲, △) before supplementation (PS), before exercise (PE) and at several time points after exercise and supplementation with D-α-tocopherol (straight line) or placebo (broken line), (mean ± SEM)

**Table 2** Plasma creatine kinase

| Creatine kinase [0.1 x U] | D-α-tocopherol | Placebo |
|---|---|---|
| Pre-supplementation | 53±5 | 56±7 |
| Pre-exercise | 51±4 | 47±4 |
| Post-exercise | | |
| + 0 h | 56±5 | 54±4 |
| + 3 h | 71±8 | 93±16 |
| + 24 h | 108±20 | 163±41 |
| + 48 h | 76±10 | 105±22 |

Values are means ± SEM, n = 13

previous study elevated concentrations of the vitamin C radical in response to exercise were detected in heart muscle of rats and attributed to an augmented oxidation of ascorbate by ROS [23]. The present investigation is the first study which analyzed plasma for the vitamin C radical in exercising humans by means of EPR. Unchanged levels of vitamin C radical and TBARS after exercise let us to suppose that our exhaustive treadmill protocol failed to induce detectable oxidative stress with respect to the measured parameters. These results may be due to the sufficient antioxidant defense mechanisms in the blood also maintained during and after the exercise regimen and being independent from vitamin E treatment. Alternatively, the parameters (TBARS) chosen might not be sensitive enough to detect moderate oxidative stress. Accordingly, Dufaux and co-workers did not observe significant changes of TBARS at any time point after exercise in 12 moderately trained subjects after a 2.5 h run [24]. Glutathione (GSH) in blood decreased significantly and oxidized GSH (GSSG) increased. In all subjects the GSH/GSSG ratio showed a marked decline after exercise. Consequently, GSH and GSSG might be more sensitive markers for oxidative stress during exercise in moderately trained subjects. If the subjects are highly trained, however, neither TBARS nor blood glutathione levels changed in response to a triathlon [25].

## Effects of D-α-tocopherol supplementation

D-α-Tocopherol is the primary lipid-soluble antioxidant and influences cellular responses to ROS including modulation of signal transduction pathways [26]. In the present investigation ingestion of 500 I. U. D-α-tocopherol daily over a period of 8 days was sufficient to induce a 2.3-fold increase in plasma lipid-standardized D-α-tocopherol which coincides with reports that chronic administration of 440 mg D-α-tocopherol resulted in steady-state plasma levels that occurred at days 4–5 of supplementation [27].

It is well established that ascorbate regenerates oxidized D-α-tocopherol by a nonenzymatic mechanism [28] resulting in the vitamin C radical. Very recently we showed an increased concentration of vitamin C radical in smokers after administration of D-α-tocopherol and ascorbate [21]. We assume that the increase of the vitamin C radical in smokers is a consequence of chronic smoke exposure-induced oxidative stress. Therefore, our results of unchanged values of vitamin C radical under administration of D-α-tocopherol may indicate a balanced antioxidant defense in plasma of the investigated subjects at rest and after the exhaustive exercise procedure. In addition, our data clearly show that vitamin E supplementation resulting in higher steady-state levels does not result in an increased concentration of vitamin C radical and consequently a more or less pro-oxidative unbalance in human plasma. As recently described, we found a high percentage of HO-1 (hemoxygenase-1)-positive monocytes at rest in the study group (vitamin E treated: (92.7 ± 3.1)%; placebo: (96.3 ± 1.1)%) [29]. Directly after exercise, 7 out of 13 subjects exhibited an insignificantly lower percentage of HO-1 positive granulocytes and mean fluorescence intensity for HO-1 when receiving D-α-tocopherol. Vitamin E might indeed exert effects on oxidative stress-induced HO-1 expression as recently documented in β-carotene-treated and UV-exposed human skin fibroblasts [30]. The enhanced expression of HO-1 following β-carotene treatment and UVA exposure was completely suppressed when the cells were pre-treated with D-α-tocopherol. These data may underline the importance of a balanced anti-oxidative network which at least contributes to the overall defense against oxidative stress, but if unbalanced may also exert pro-oxidant effects.

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