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TOCOPHEROL ACETATE *VS.* OXIDATIVE STRESS INDUCED BY PHYSICAL EXERCISE IN RATS

Anna Gronowska-Senger, Magdalena Górnicka, Katarzyna Kołodziejska

Department of Human Nutrition, Chair of Nutrition Assessment, Faculty of Human Nutrition and Consumer Sciences, Warsaw University of Life Sciences, Warsaw, Poland

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The study was aimed at determining the effect of various doses of tocopherol acetate on the oxidative stress induced by physical exercise in rats. The experiment was conducted on growing Wistar male rats. The oxidative stress was induced by running on a treadmill with tape movement velocity of 20 m/min for 15 min for the period of 10 days. After that period, samples of rats serum were determined for levels of α-tocopherol and lipid peroxides as well as for antioxidative potential and α-tocopherol content of liver.

The study demonstrated that physical exercise was leading to enhanced release of tocopherol from liver to blood, depending on the applied dose of the vitamin. The serum level of lipid peroxides was lower in the animals subjected to the training and inversely proportional to the dose of tocopherol. In the animals not subjected to exercise, the oxidative stress was observed to be enhanced at the highest dose of the vitamin.

INTRODUCTION

In the last 90 years, namely since the discovery of vitamin E, a number of investigations have been conducted in order to describe its metabolic functions. Vitamin E has been shown to be capable to protect lipid against oxidation, to act as an antioxidant against the other cell constituents, and – potentially – to affect gene expression [Clycombe & Meydani, 2001; Zinng, 2007]. The antioxidative action of α-tocopherol is linked, first of all, with its participation in the protection of cellular membranes against effects of detrimental factors [Bansal *et al*., 2005; Traber & Atkinson, 2007]. By entering into reactions with free radicals, α-tocopherol protects protein and long-chain unsaturated fatty acids present in cellular membranes against oxidation [Antosiewicz *et al*., 2002], acts as a scavenger of free radicals and prevents the peroxidation reaction of lipids, thus protecting cells and sub-cellular structures by sustaining their biological functions [Traber & Atkinson, 2007]. Tocopherol quenches singlet oxygen and reacts with superoxide anion radical and hydroxyl radical as well as with nitric oxide radicals and ozone [Sroka *et al.,* 2005]. The function of α-tocopherol as an antioxidant consists in its oxidation to a tocopheroxyl radical (α-T$^{\bullet}$), through the acceptance of an electron from RO_2, owing to which a more reactive $RO_2^{\bullet}$ is being quenched. A tocopherol radical is relatively stable and, due to its relatively low reactivity, does not attack other molecules. It may instead react with another free radical, forming an inactive product with it. This way a single molecule of α-tocopherol is capable of eliminating two free radicals [Stahl, 2002]. Physical exercise is linked with an enhanced production of reactive forms of oxygen, thus increasing the utilization of antioxidative vitamins, including α-tocopherol [Faff, 2003; Mastaloudis *et al.*, 2004; Ramel *et al*., 2004; Aquilo *et al*., 2005].

The effectiveness of α-tocopherol in the reduction of oxidative stress has not been explicitly elucidated yet, especially in respect of its dose. Studies have shown that, on the one hand, the highest doses were the most effective both in humans and animals [Avellini *et al*., 1999; Sacheck *et al*., 2000, Jordão *et al*., 2004], yet on the other hand – long-term supplementation with high doses of vitamin E yielded an opposite effect [Hathcock *et al*., 2005; Bjelakovic *et al*., 2007]. Thus, it seemed interesting to undertake investigations aimed at determining the effectiveness of various doses of tocopherol in the reduction of oxidative stress induced by physical exercise.

MATERIAL AND METHODS

The experiment was conducted on a group of 49 male Wistar rats with the initial body weight of 77±4 g. The animals were placed individually in steel cages, in a ventilated room with a constant temperature of 24±3°C, and 12-h light/darkness cycle. Body weight of the rats was controlled every second day, and diet intake – every day. The protocol of the experiment was approved by the Local Commission of Ethics.

In the adaptation period (3 day), the animals were receiving water *ad libitum* and a diet without vitamin E [Reeves, 1997]. The composition of the diet was presented in Table 1. On termination of that period, four rats were anaesthetized, and samples of their blood and liver were collected to determine the initial level of α-tocopherol. The other animals

Author's address for correspondence: Prof. Dr. Anna Gronowska-Senger, Department of Human Nutrition, Warsaw University of Life Sciences, ul. Nowoursynowska 159c, 02-776 Warsaw, Poland: e-mail: anna_gronowska_senger@sggw.pl

TABLE 1. Composition of an experimental diet.

Component	Content (g/100 g)
Wheat starch	53
Casein	20
Saccharose	10
Lard	7
Potato starch	5
Mineral mix	4
Vitamin mix	1
L-methionine	0.3
Choline	0.3

were divided into groups according to a scheme provided in Table 2. The control groups (C) included 5 rats, whereas the experimental ones (E) – 6 animals each. The animals had an unrestricted access to water and feed mixtures.

In order to induce the oxidative stress, the animals from selected groups (Table 2) were running every day on a treadmill with the speed of 20 m/min for 15 min for 10 days of the exact experiment. α-Tocopherol acetate in three various doses (Table 2) was administered *per os* with a standardized dropper in a dose of 2 drops 20 min before running to all animals from the experimental groups. Solutions of tocopherol were prepared with the use of a pharmaceutical preparation of tocopherol acetate in peanut oil with a concentration of 300 mg/mL (TERPOL). Assuming that 1 mg of tocopherol acetate is equal to 0.91 mg of α-tocopherol, the preparation was diluted with soybean oil (taking into account vitamin E contained in it), so as to obtain: 0.25, 0.5 and 2.0 mg of α-tocopherol in 1 drop (25 μL). Owing to a negligible content of the oil in the dose administered to rats, its contribution was not considered in the energy value of diet.

On termination of the experiment, the animals were weighed, anaesthetized intramuscularly with a mixture of ketamine and xylazine, and samples of their blood and liver were collected for analyses. Blood was centrifuged in order to obtain serum, which was then immediately frozen until analyses. Livers were weighed, rinsed in physiological saline and frozen until analyses.

The level of α-tocopherol was determined in blood serum and liver with the HPLC-UV method [Van Vliet, 1991]. Determinations were conducted on a GILSON chromatograph with a UV-VIS detector and a HYPERSIL RP-C 18 chromatographic column (4.6 × 150 mm, 5 μm). The mobile phase was a mixture of acetonitrile, hexane and isopropanol (65:14:21), delivered at a flow rate of 0.8 mL/min. The measurement was performed at a wave length of UV 295 nm.

TABLE 2. Experimental model.

Group	Tocopherol dose (mg/day/rat)
CE*	-
E_1E	0.5
E_2E	1.0
E_3E	4.0
CnE	-
E_1nE	0.5
E_2nE	1.0
E_3nE	4.0

*C – denotes control groups, E – denotes groups subjected to physical exercise, nE – denotes groups not subjected to physical exercise

Prior to the exact determinations, the samples were prepared as follows: 2 mL of 98% ethanol with BHT as an antioxidant were added to 1 mL of serum and the sample was shaken for 30 s. After 15 min, 1 mL of chloroform was added to the mixture, which was next shaken again for 2 min. The extract obtained was centrifuged (1500 x*g*, 10 min) and the clear supernatant collected was evaporated under nitrogen atmosphere.

The samples of liver were homogenized, and next supplemented with 2 mL of 98% methanol with BHT as an antioxidant. The mixture was shaken for 30 s. After 10 min, 4 mL of chloroform were added and the mixture was shaken again for 4 min. The suspension obtained was centrifuged (600 x*g*, 10 min), and the clear supernatant collected was evaporated under nitrogen atmosphere.

So prepared samples were dissolved in 0.2 mL of hexane and injected onto the column. Results obtained were referred to standard curves plotted for α-tocopherol (Sigma): 1-10 μg/mL for serum and 0.2-4 mg for liver.

The antioxidative potential of blood serum was determined with the colorimetric ABTS method [Re *et al*., 1999], which consists in the measurement of a decrease of absorbance of $ABTS^{(+}$ cation radical being proportional to the content of antioxidants present in a sample. Such antioxidants as ascorbate and glutathione respond rapidly and their assay in a short time span (10 s) is a measure of their content in a sample. In turn, other antioxidants respond more slowly and their measurement after a longer period of time is a measure of the content of the so-called "slow antioxidants" [Bartosz, 2006]. For this reason, assays were carried out firstly for the initial absorbance (A0), and then exactly 10 s after 20 μL of the analysed serum has been added (A1). After 30 min, the measurement of absorbance was repeated once more (A2). Results obtained were converted according to absorbance values of a standard solution of Trolox.

Lipid peroxides in serum were determined with the TBARS method which consists in the measurement of absorbance of malondialdehyde (MDA) – a substance being one of the end products in reactions of lipids oxidation, reacting with thiobarbituric acid (TBA) at a wave length of 532 nm. Results obtained were referred to a standard curve plotted against 1,1,3,3-tetraethoxypropane (TEP) and expressed in nmoles of TEP [Jentzsch *et al*., 1996].

Results obtained in the study were subjected to an analysis of variance (ANOVA). In order to determine correlations between tocopherol dose and the analysed biomarkers, use was made of an analysis of regression (Statgraphics Plus 5.1). In both cases, a significance level was adopted at α=0.05.

RESULTS

No significant differences were demonstrated in the mean daily diet intake and in the mean body weight gain in groups

receiving various doses of tocopherol (Table 3). Still the values of body weight gains in the groups subjected to physical exercise were higher as compared to those reported for the non-exercising groups, except for control groups not receiving vitamin E – in the case of which the values were similar.

The level of α-tocopherol in serum and liver was positively correlated with the dose of tocopherol acetate in all groups examined (Figures 1 and 2). The mean level of α-tocopherol in the control group subjected to physical exercise was significantly lower (p=0.00) as compared to the control group not subjected to training on a treadmill.

TABLE 3. Mean values (±SD) of body weight and mean (±SD) diet intake in the groups examined.

Group	Initial body weight (g)	Final body weight (g)	Body weight gain (g)	Diet intake (g/d)
CE	91.9±5.9	159.0±9.5	67.1±6.8	13.6±1.98
E_1E	92.5±5.7	150.1±8.8	61.2±8.2	14.5±0.92
E_2E	92.6±6.2	148.3±7.0	55.6±9.5	14.1±1.20
E_3E	93.4±7.1	148.4±8.4	55.9±8.4	14.5±1.03
CnE	91.5±6.5	155.1±8.5	63.6±8.1	13.4±1.42
E_1nE	92.8±5.6	135.3±10.5	43.0±6.6	14.6±1.28
E_2nE	93.5±7.2	144.0±13.5	50.4±8.4	14.7±1.04
E_3nE	91.5±5.2	143.3±11.2	50.0±5.9	15.3±1.45

The content of α-tocopherol in serum was observed to increase along with an increasing dose of tocopherol acetate both in the exercising and non-exercising groups, however, its values determined in the groups subjected to physical exercise were significantly higher than in the non-exercising groups. In the exercising groups, the higher level of α-tocopherol in the serum was linked with its lower concentration in the liver (Figure 2), whereas in the non-exercising groups an opposite phenomenon was observed, *i.e.* a lower level of α-tocopherol in the serum corresponded with its higher concentration in the liver.

In addition, in the exercising and non-exercising groups receiving 1 mg of tocopherol acetate the analyses demonstrated the greatest increase in the level of α-tocopherol in the liver, and the lowest one – in the serum (Table 4). An opposite dependency was, in turn, observed for tocopherol doses of 0.5 and 4 mg. As compared to the control groups (both the exercising and non-exercising ones), the greatest increase in α-tocopherol content of liver and serum was reported upon administration of tocopherol dose of 4 mg. The increase in the concentration of vitamin E in liver was greater in the non-exercising groups, whereas in the serum higher values were recorded in the exercising groups.

In analysing the effect of tocopherol acetate dose on the oxidative stress (Table 5), expressed by the value of an antioxidative potential (dependent on "rapid" and "slow" antioxidants), a positive correlation was observed between its dose and the antioxidative potential dependent on the "slow" antioxidants in groups subjected to physical exercise. Along with an increasing dose

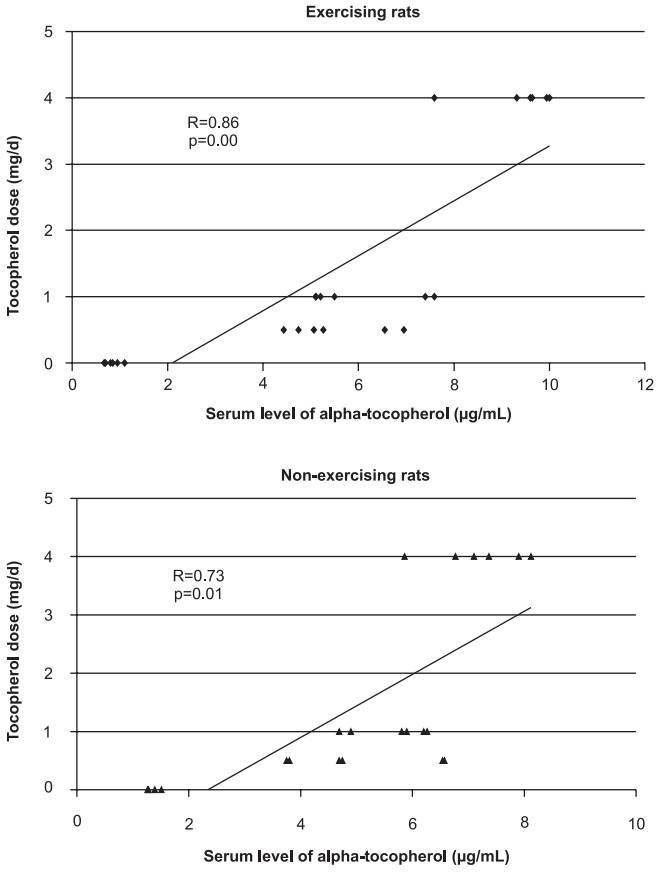

FIGURE 1. Correlations between tocopherol acetate dose and its serum level.

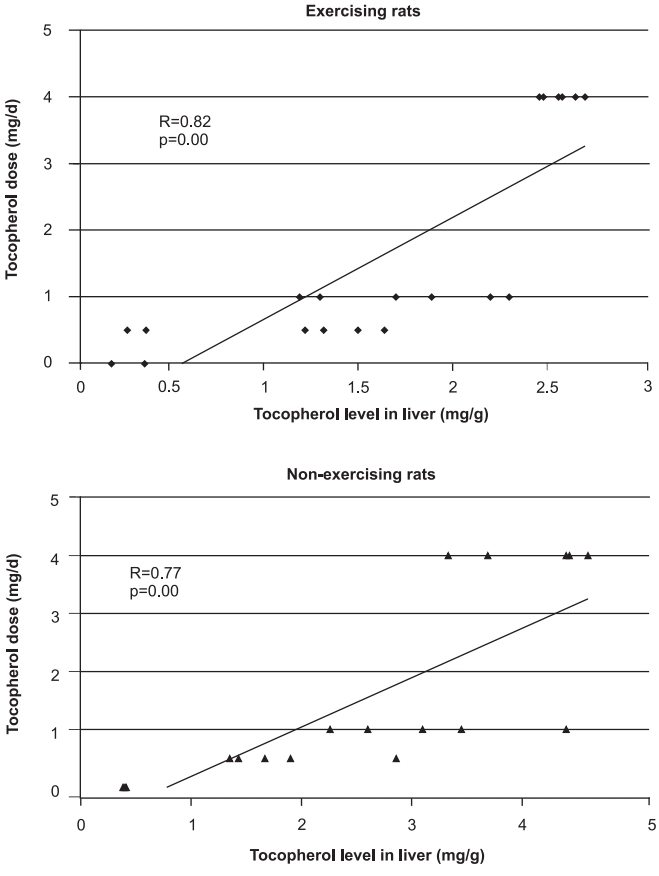

FIGURE 2. Correlations between tocopherol acetate dose and its level in liver.

TABLE 4. Changes in contents of tocopherol in serum and liver.

	0	CE	CnE	E_1E	E_2E	E_3E	E_1nE	E_2nE	E_3nE
Liver (μmol/mg)									
Differences between groups		- 0.59	- 0.30	0.25	2.94	1.65	2.35	5.10	1.03
CE *vs*. EE and CnE *vs*. EnE				0.25	3.19	4.84	2.35	7.44	8.48
EE *vs*. EnE							2.35	4.50	3.89
Serum (μmol/mL)									
Differences between groups		-0.002	- 0.003	0.009	0.001	0.010	0.006	0.004	0.004
CE *vs*. EE and CnE *vs*. EnE				0.009	0.009	0.019	0.006	0.009	0.013
EE *vs*. EnE							- 0.001	0.002	- 0.005

TABLE 5. Indices of oxidative stress in rats administered various doses of tocopherol.

	Groups									
	CE	E_1E	E_2E	E_3E	R*	CnE	E_1nE	E_2nE	E_3nE	R*
Antioxidative potential (μmol Trolox/mL)										
"Rapid"antioxidants	50.05^{e} ±2.0	$32.63^{a.c.d}$ ±2.0	$32.21^{a.c.d}$ ±1.7	$31.37^{a.c.d}$ ±1.9	-0.52	39.06^{e} ±1.2	$36.20^{b.c}$ ±0.9	36.99^{c} ±0.5	38.95^{c} ±0.7	0.15
"Slow" antioxidants	69.69^{e} ±2.4	$85.33^{a.c.d}$ ±0.5	$85.96^{a.c.d}$ ±1.3	$87.66^{a.c.d}$ ±1.2	0.60	80.85^{e} ±1.8	$82.49^{b.c}$ ±1.4	$82.78^{b.c}$ ±0.8	$82.22^{b.c}$ ±0.8	0.39
Lipid peroxides (nmol/mL)										
	3.32^{e} ±0.79	$8.76^{a.c.d}$ ±0.96	$8.98^{a.c.d}$ ±0.40	$5.62^{a.c.d}$ ±0.12	-0.47	5.43^{e} ±0.91	$5.60^{c.f}$ ±0.28	$11.62^{b.c.f}$ ±0.72	$14.07^{b.c.f}$ ±0.25	0.68

R*- correlation coefficient; [a] p≤0.05 as compared to CE; [b] p≤0.05 as compared to CnE; [c] p≤0.05 between EE and EnE groups; [d] p≤0.05 between EE groups; [e] p≤0.05 between control groups; [f] p≤0.05 between EnE groups.

of tocopherol acetate, the antioxidative potential of blood serum was observed to increase significantly. In contrast, in the case of "rapid" antioxidants, dependencies between tocopherol dose and the value of the potential in the exercising groups were inversely proportional, whereas in the non-exercising groups – directly proportional, but statistically insignificant.

The level of α-tocopherol was positively correlated with the antioxidative potential dependent on the "slow" antioxidants in all groups examined (Figure 3). The exercising groups were characterised by a higher level of α-tocopherol in the serum and, simultaneously, by higher values of the antioxidative potential (as compared to the non-training group). In contrast, in the non-training groups, the lower level of α-tocopherol in the serum corresponded with a lower potential (as compared to the exercising groups). In contrast, the correlation between the antioxidative potential of the serum linked with the action of the "rapid" antioxidants and the level of α-tocopherol in blood plasma (Figure 4) was negative (r=-0.83), especially in respect of the exercising groups in the case of which it was statistically significant (p≤0.05).

The level of lipid peroxides in the groups of rats subjected to physical exercise (Table 5) was negatively correlated with the dose of tocopherol. In the case of the non-training animals, a lipid peroxidation index was increasing significantly along with an increasing dose of tocopherol. In turn, no correlation was noted between the level of α-tocopherol in the serum and the content of lipid peroxides (Figure 5), both in the exercising and non-exercising groups (p≥0.05). In the groups not subjected to physical exercise, the positive coefficient of correlation (r=0.58) attained higher values as

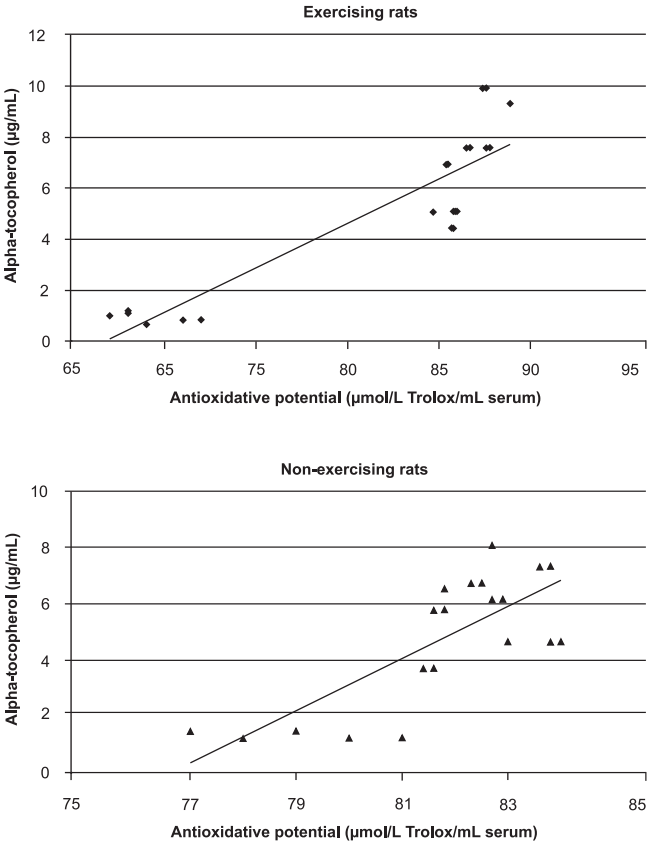

FIGURE 3. Correlations between serum level of α-tocopherol and the antioxidative potential of serum dependent on the "slow" antioxidants.

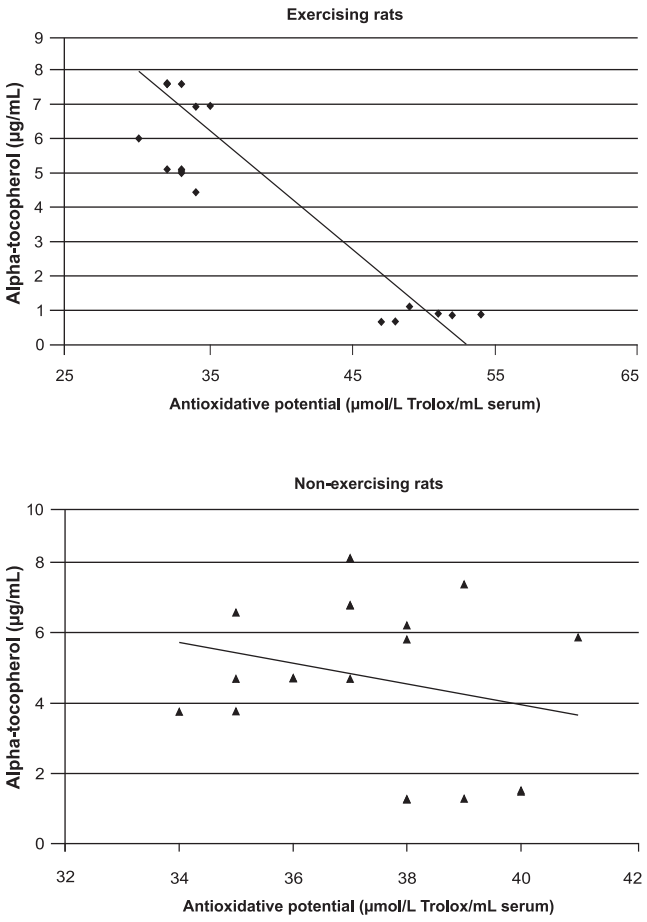

FIGURE 4. Correlations between serum level of α-tocopherol and the antioxidative potential of serum dependent on the "rapid" antioxidants.

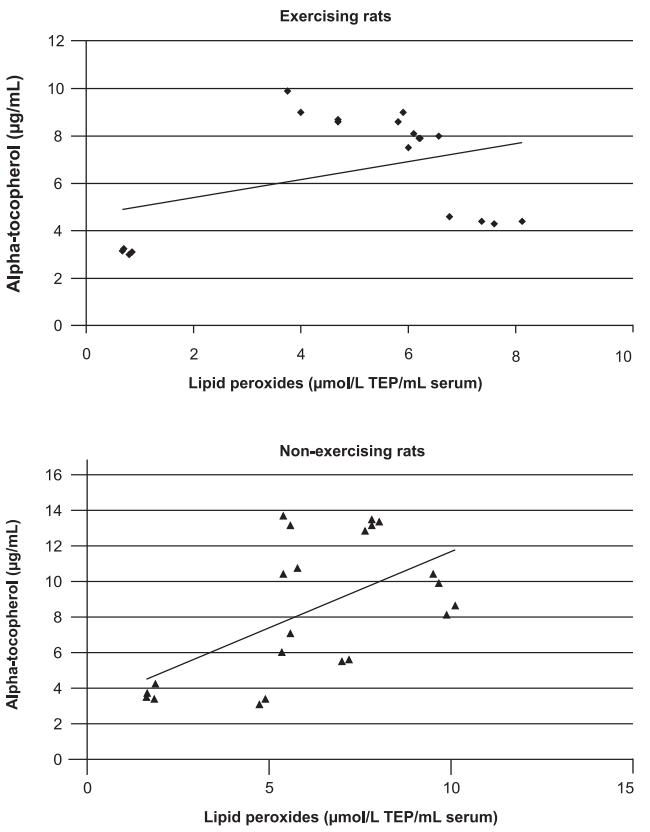

FIGURE 5. Correlations between serum level of α-tocopherol and the level of lipids peroxides.

compared to the exercising groups, the first groups were also characterized by a higher level lipid peroxides upon tocopherol treatment at doses of 1 mg and 4 mg.

DISCUSSION

Results obtained in the reported study confirmed the protective effect of tocopherol against free radicals, for the physical exercise was leading to an increase in α-tocopherol level in the serum and its decrease in liver. Avellini *et al.* [1999] and Mastaloudis *et al.* [2004] explain such changes with an increasing demand for antioxidants under conditions of oxidative stress, which results in the enhanced transfer of α-tocopherol from tissues to blood, including its release from free fatty acids accumulated in fatty tissue or an increased flow of fatty acids through liver being stimulated by the secretion of VLDL, which tocopherol is built into through the Tocopherol Transfer Protein (TTP). The increased level of tocopherol in the serum is also likely to be due to rapid redistribution from tissues as a result of the enhanced production of free radicals [Deaton & Marlin, 2003].

The release of α-tocopherol from liver and the increase in its concentration in the serum, observed in the presented study upon physical exercise, were due to the enhanced mobilization of antioxidative systems dependent on the "slow" and "rapid" antioxidants, including uric acid, ascorbic acid or bilirubin, which act as "supportive" mechanisms before other defensive mechanisms and enzymes are activated. The reported decrease in the antioxidative potential dependent on the "rapid" antioxidants, corresponding to an increasing level of α-tocopherol in the serum of rats exposed to physical exercise, confirms the co-action of the entire system of antioxidants, especially the synergism with vitamin C. The tocopheroxyl radical formed upon the action of reactive forms of oxygen is reduced by ascorbic acid again to α-tocopherol, owing to which its level in the serum remains high, and that of other antioxidants is observed to decrease. Analogously, the higher level of the total antioxidative potential and that of the antioxidative potential dependent on the "rapid" antioxidants determined in the non-exercising groups indicates that these compounds were not involved into the reduction process of the tocopheryl radical. Those results confirm the key significance of the whole system composed of low-molecular antioxidants to biological functions of vitamin E [Traber & Atkinson, 2007]. It has also been shown in a study by Hill *et al.,* in which deficiency of vitamin E accompanied by deficiency of selenium [Hill *et al*., 2001] or by deficiency of vitamin C [Hill *et al.*, 2003] caused death of guinea pigs.

On the one hand, the physical exercise generates the excess of free radicals, thus leading to *e.g.* oxidative damage to muscles as a result of the enhanced peroxidation of lipids, but on the other hand epidemiological data indicate that it minimizes the risk of the incidence of diseases induced by

the oxidative stress. That phenomenon results from adaptation processes of a body proceeding at a molecular level and linked with the maintenance of homeostasis. The adaptation mechanism is initiated by transcription factors that evoke an increase in the activity of antioxidative systems, including: systems of repair/elimination of oxidative damages, increased resistance to oxidants and, consequently, a smaller extent of oxidative damages [Radak, 2008]. Taking all these into account, higher values of lipid peroxides were expected in the exercising groups not receiving tocopherol. Surprisingly, the results obtained were opposite, *i.e.* the lowest level of lipid peroxides was determined in the control exercising group (CE), which corresponds with findings reported by Somani *et al*. [1995], who when analysing values of oxidative stress indices in training and non-training rats showed a lower degree of lipids peroxidation in the animals in which the physical exercise was preceded by training, at simultaneously higher initial level of glutathione and enhanced initial activity of antioxidative enzymes as compared to the non-training group. On that basis it may be speculated that the low level of peroxides in the control exercising group (CE) resulted from an enhanced activity of antioxidative systems linked with the action of enzymes and low-molecular "supportive" antioxidants, which could be indicated by the highest values of the potential dependent on the "rapid" antioxidants. The highest levels of lipid peroxides were determined in the groups not subjected to physical exercise but, simultaneously, receiving the higher dose of tocopherol acetate (1 mg/d and 4 mg/d). It points to the prooxidative effect of α-tocopherol in situation when it is not involved in defensive mechanisms of the body against detrimental effects of free radicals, but exhibits the capability to reduce Fe(III) ions to Fe(II) and Cu(II) ions to Cu(I) in Fenton's and Haber-Weis' reactions in which it stimulates the generation of free radicals $OH^{\bullet}$ [Sroka *et al*., 2005]. That effect was also described by Schneider *et al*. [2003] and McAnulty *et al*. [2005], who demonstrated the prooxidative effect of tocopherol in respect of fatty acids. The action of vitamin E is determined by physiological processes [Schneider, 2005]. Though its most commonly-known antioxidative function has not been thoroughly elucidated yet [Azzi, 2007], ample studies indicate that it may also display prooxidative activity.

Summarizing the own research, the factor that determined the extent of a reduction in oxidative stress induced by physical exercise turned out to be the enhanced release of α-tocopherol from liver to blood and the increase in the antioxidative potential of serum dependent on the "slow" antioxidants (including α-tocopherol). For this reason, the animals subjected to physical exercise and simultaneously receiving α-tocopherol as an antioxidant were exposed to the detrimental effects of free radicals to a lesser extent. The enhanced mobilization of α-tocopherol in those groups caused that the level of lipid peroxides in the serum did not increase as compared to the initial level, whereas in the non-exercising rats α-tocopherol exhibited prooxidative properties, which was manifested in the intensified degree of lipid peroxidation.

Increasing the dose of α-tocopherol in the groups subjected to physical exercise was leading to a more efficient reduction oxidative stress. The increasing dose of tocopherol acetate was accompanied by higher levels of the antioxidative potential dependent on the "slow" antioxidants and by a lower degree of lipids peroxidation.

CONCLUSIONS

1. Tocopherol exhibits antioxidative activity upon oxidative stress induced by physical exercise, yet the mechanism of its action requires further extensive research.

2. The excessive supply of tocopherol at an elevated oxidative stress poses a risk of its prooxidative action.

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