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Alpha-Tocopherol May Protect Hepatocytes Against Oxidative Damage Induced by Endurance Training in Growing Organisms

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article

Abstract

Background. Training-induced oxidative stress can be reduced by α-tocopherol. Adequate intake of α-tocopherol could have health benefits for previously untrained young subjects.
Objectives. The aim of this study was to determine the effects of training and different doses of α-tocopherol on exercise-induced oxidative stress in rat livers.
Material and Methods. Young male Wistar rats (n = 40) were randomly divided into eight groups (undergoing training and not undergoing training, given orally administered α-tocopherol doses of 0, 0.5, 1.0 and 4.0 mg). Every day for 10 consecutive days, the rats in the training groups ran for 15 min on a treadmill at 20 m/min to induce oxidative stress. Hepatic oxidative stress was evaluated based on the liver concentrations of 8-hydroxy-2'-deoxyguanosine (8-OHdG) and thiobarbituric acid reactive substances (TBARS).
Results. The liver concentrations of α-tocopherol were significantly influenced by α-tocopherol doses ($p < 0.001$) and physical exercise ($p < 0.001$). The liver concentrations of α-tocopherol increased in response to the highest dose (4 mg/d) of α-tocopherol in the non-training groups. In the training groups, the liver concentrations of α-tocopherol were independent of the dose. The levels of TBARS, a marker of lipid peroxidation, were lowest in the training and non-training rats administered 4.0 mg of α-tocopherol. Physical exercise and α-tocopherol doses significantly influenced TBARS concentrations ($p = 0.004$, $p < 0.05$).
Conclusions. The results of this study indicate that running training causes lipids peroxidation and reduces α-tocopherol levels in the liver, but it does not contribute to DNA damage. Increased liver concentrations of α-tocopherol were found to exert a protective effect against oxidative damage induced by endurance training. An adequate intake of α-tocopherol is important for previously untrained young subjects (**Adv Clin Exp Med 2016, 25, 4, 673–679**).

Key words: oxidative stress, rats, 8-OHdG, α-tocopherol, TBARS.

Regular physical exercise is known to provide health benefits, but the molecular mechanisms that are responsible for the effects of exercise on peripheral tissues have not yet been fully explained. Skeletal muscles play a key role during exercise, but other organs, such as the liver, are important in the maintenance of homeostasis. The liver is a complex organ that performs many functions, and its main role during exercise is maintaining normal blood glucose levels and supplying lipids as energy substrates for working muscles [1]. Recent research indicates that the hepatic response to strenuous exercise activates the mitogen-activated protein kinase (MAPK) and interleukin-6 (IL-6) cytokine signaling pathways, which results in marked transcriptional upregulation of stress response genes and genes activated by energy depletion. After exercise, lower plasma glucose levels in-

crease the expression of stress response genes [2]. Oxidative and detoxifying processes, as well as free radical reactions, take place in hepatocytes. During exercise, the oxygen demand of muscles and oxygen consumption increase, which is why exercise is associated with higher production of free radicals [1–3].

Reactive oxygen species (ROS) can directly cause oxidative damage to DNA and lipids. As Huang et al. wrote: "Oxidative damage to DNA may result in base modification, sugar damage, strand break and DNA-protein cross-links. Of these, modification of guanine by hydroxyl radicals at the C-8 site, frequently estimated as [8-Oxo-2'-deoxyguanosine (8-OHdG)], is the most commonly studied lesion" [4] and one of the most widespread types of oxidative damage to DNA [5, 6]. The liver appears to be more sensitive to oxidative stress than other tissues [7], but research studies often deliver ambiguous results. Choi and Cho [8] and Liu et al. [9] reported that endurance training did not affect oxidative stress in the liver, whereas Taysi et al. [10] demonstrated that exhaustive exercise decreased the levels of lipid peroxidation in the livers of rats undergoing training.

Many biomonitoring studies have investigated the potential of dietary antioxidants to reduce oxidative damage to DNA and lipids. One of them is α-tocopherol, which can inhibit free radical formation and has protective effects on lipids. Moreover, α-tocopherol reduces chromosomal damage induced by free radicals and activates endonucleases to increase the rate of DNA repair [11]. Avellini et al. [12], Sacheck et al. [13] and Jardão et al. [14] demonstrated that tocopherol's ability to reduce oxidative stress is determined by its dose, and that the highest dose is most effective in both humans and animals. The liver is an important organ for α-tocopherol metabolism because it is responsible for maintaining normal plasma levels of α-tocopherol [15].

There is no unambiguous data concerning oxidative tissue damage in young animals or the benefits of α-tocopherol supplementation for animals that are not undergoing training. The effects of physical activity have been investigated in different models, but most studies have focused on adults, aging animals, healthy adults or patients affected by several disorders.

This study was designed to test the hypothesis that endurance training in young and previously untrained animals causes oxidative damage that can be reduced by high doses of α-tocopherol. The present authors' previous research revealed that dietary supplementation with 2 mg of α-tocopherol increased plasma levels of α-tocopherol after exercise in growing rats and reduced plasma concentrations of thiobarbituric acid reactive substance (TBARS) [16, 17]. The liver is a key organ that generates free radicals, stores fat-soluble vitamins, synthesizes vitamin C and controls redox homeostasis. The aim of this study was to determine the effect of training and different doses of α-tocopherol on exercise-induced oxidative stress in rat livers. This goal was achieved by analyzing the influence of different α-tocopherol doses on the concentrations of α-tocopherol, lipid peroxides and 8-OHdG in rat livers.

Material and Methods

The Animals

The experiment was carried out on male Wistar rats (n = 40) with an initial body weight of 77 ± 4 g. The rats were housed individually in steel cages in a temperature-controlled room with constant humidity and a 12 h/12 h light/dark cycle. The animals had free access to water and food, consisting of the American Institute of Nutrition rodent diet for adult maintenance (the AIN-93M diet), but without α-tocopherol (Table 1) [18]. All the procedures were carried out in compliance with ethical standards and were approved by the Third Local Ethics Committee for Animal Experiments at the Warsaw University of Life Sciences (WULS–SGGW, Warszawa, Poland).

The rats were divided into eight groups of five animals each: Four groups of animals that underwent training and received orally administered α-tocopherol in doses of 0, 0.5, 1.0 and 4.0 mg; and four groups of non-training animals that were orally administered α-tocopherol doses of 0, 0.5, 1.0 and 4.0 mg. Tocopherol acetate (a concen-

Table 1. The composition of the modified AIN-93M diet used in the experiment

Ingredients	Content, %
Caseine	14.0
Cornstarch	46.5
Dextrose	15.5
Sucrose	9.0
Cellulose	5.0
Soybean oil	4.0
t-Butylhydroquinone	0.0008
Mineral mix	3.5
Vitamin mix*	1.0
L-cystine	0.18
Choline bitertrate	0.25
Supplement (#410950)	1.0

* without α-tocopherol.

tration of 300 mg/mL; Medana Pharma Terpol Group S.A., Sieradz, Poland) was diluted with soybean oil. The α-tocopherol was administered orally in drops, between 9:00 and 11:00 am every day, 20 min before the exercise sessions; the animals in the two control groups received soybean oil drops. The AIN-93M diet established 1.0 mg as the daily requirement of α-tocopherol, so the doses used in the study ranged from half (0.5 mg) to four times (4.0 mg) that amount.

Over a period of 10 days, the rats from the four training groups ran on a treadmill with electrical stimulation at a rate of 20 m/min for 15 min per day.

Liver Tissue Sampling and Testing

At the end of the experiment, the animals were anesthetized with intramuscular ketamine and xylazine. The livers were isolated, rinsed in ice-cold saline to remove blood and tissue remnants, weighed and frozen until the analyses. They were homogenized in Tris-HCl buffer. The concentrations of α-tocopherol, lipid peroxides and 8-OHdG in the liver homogenates were determined.

The liver concentrations of α-tocopherol were determined by the HPLC-UV method [19, 20]. Following the addition of 10% ascorbic acid solution as an antioxidant, α-tocopherol was extracted by hexane and ethanol. After centrifugation, clear supernatant was collected and evaporated under nitrogen. Samples were dissolved in hexane and injected onto the LiChroCART® 250-4 LiChrospher® 100 RP-18 (5 μm) column (Merck KGaA, Darmstadt, Germany).

Acetonitrile-hexane-isopropanol (65:14:21, v/v/v) was applied as the eluent. The flow rate was 0.8 mL/min.

Lipid peroxide levels were determined by the thiobarbituric acid reactive substance (TBARS) method [21], involving spectrophotometric determination of the concentrations of malondialdehyde (MDA), one of the final products of lipid oxidation, reacting with thiobarbituric acid (TBA).

The concentrations of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in the liver were measured by the modified Foksiński method [22] using high-performance liquid chromatography – ultraviolet and electrochemical detectors (HPLC/UV/ECD). DNA hydrolysates were analyzed by isocratic elution chromatography using 25 mM sodium acetate, 12.5 mM citrate (pH 5.0)-methanol (88:12, v/v). Deoxyguanosine was detected at 254 nm. The concentrations of 8-OHdG in the liver were determined with an electrochemical detector: Guard cell +750 mV, detector 1: +130 mV (as a screening electrode), detector 2: +450 mV (as a measuring electrode set to a sensitivity of 20 nA/V). The procedure was described in detail by Wawrzyniak et al. [16].

Statistical Analysis

The statistical analysis was carried out using STATISTICA v. 10 software (StatSoft Inc., Tulsa, USA). The normality of the data distribution was tested using the Shapiro-Wilk test. The data were subjected to a two-way analysis of variance (ANOVA) to determine the influence of α-tocopherol, training and two-way interactions on the variables measured (α-tocopherol, lipid peroxide and 8-OHdG concentrations in the liver). Significant interactions identified by the ANOVA were subjected to a *post-hoc* Tukey's test to compare group means. Pearson's linear correlation coefficient (r) was calculated to analyze the strength of the relationships between individual variables. Statistical significance was set at $\alpha = 0.05$. The results were expressed as means and standard deviation.

Results

No significant differences were found between the groups in terms of the body weights of the animals or total feed intake at the beginning and end of the experiment (data not given).

Changes in α-Tocopherol Levels

The concentrations of α-tocopherol in the animals' livers (Table 2) were significantly affected by α-tocopherol doses ($p < 0.001$) and exercise ($p < 0.001$). The concentrations of α-tocopherol increased in response to the highest dose (4 mg/d) of α-tocopherol in the non-training groups. In the training groups, α-tocopherol levels in the liver were independent of the dose.

Changes in TBARS Concentrations

The concentrations of TBARS (Table 3), a marker of lipid peroxidation, were lowest in the training and non-training rats administered 4 mg/d of α-tocopherol. Both exercise and the α-tocopherol dose significantly influenced TBARS levels ($p = 0.001$ for training, and $p < 0.001$ for α-tocopherol dose). The changes in TBARS concentrations were less pronounced in the non-trained groups.

Table 2. The effects of training and α-tocopherol doses on the concentration of α-tocopherol in the liver (nM/g)

Doses of α-tocopherol (mg/day) – factor A	Training – factor B		Two-way ANOVA		
	training	non-training	factor	F	p-value
0	30.1 ± 6.0	39.2 ± 7.6[a]	A	7.876	< 0.001
0.5	29.8 ± 7.1	37.4 ± 6.0[a]	B	22.143	< 0.001
1.0	35.4 ± 7.4	48.3 ± 13.0[a, b]	A × B	1.179	0.333
4.0	39.5 ± 10.6	60.0 ± 7.3[b]			

[a, b] statistically significant differences.

Table 3. The effects of training and α-tocopherol doses on the TBARS concentration in the liver (nM/g)

Doses of α-tocopherol (mg/day) – factor A	Training – factor B		Two-way ANOVA		
	training	non-training	factor	F	p-value
0	13.0 ± 2.4[a]	10.6 ± 1.1[a]	A	8.083	< 0.001
0.5	11.3 ± 1.5[a]	10.0 ± 1.7[a]	B	14.520	< 0.001
1.0	11.0 ± 1.5[a]	9.3 ± 0.8[a, b]	A × B	0.239	0.866
4.0	9.5 ± 1.3[b]	7.6 ± 1.4[b]			

[a, b] statistically significant differences.

Table 4. The effects of training and α-tocopherol doses on 8-OHdG levels in the liver (8-OHdG/10^6 dG)

Doses of α-tocopherol (mg/day) – factor A	Training – factor B		Two-way ANOVA		
	training	non-training	factor	F	p-value
0	7.0 ± 2.3	6.5 ± 2.3	A	0.781	0.513
0.5	5.9 ± 1.2	5.5 ± 1.3	B	0.608	0.441
1.0	6.2 ± 0.9	5.5 ± 1.9	A × B	0.034	0.992
4.0	5.9 ± 2.3	5.7 ± 1.4			

The highest α-tocopherol concentrations (Table 2) and the lowest TBARS levels (Table 3) were observed in in the livers of rats administered 4.0 mg of α-tocopherol, in both the training and non-training groups.

Changes in Hepatic 8-OHdG Levels

Hepatic 8-OHdG levels (Table 4) were not influenced by the α-tocopherol dose or training ($p > 0.05$). The interaction between exercise and α-tocopherol dose had no significant effect on oxidative stress markers in the liver.

An analysis of correlations between α-tocopherol dose and the concentrations of α-tocopherol, TBARS and 8-OHdG in the liver (Table 5) revealed that the dose was negatively correlated with TBARS levels and positively correlated with α-tocopherol concentrations. Training induced the opposite effect by increasing TBARS concentrations and reducing α-tocopherol concentrations in the liver. Hepatic OHdG levels were not affected by either the administered dose of α-tocopherol or the training. Both TBARS concentration and 8-OHdG levels in the liver were strong negatively correlated with α-tocopherol concentrations in the liver (Table 5).

Discussion

This study analyzed the effect of exercise and various α-tocopherol doses on oxidative stress, measured by the concentrations of α-tocopherol, TBARS and 8-OHdG in rat livers. The main findings of the study were that physical exercise increases lipid peroxidation in the liver, and that high levels of α-tocopherol in the liver reduce hepatic oxidative damage. Exercise also lowered α-tocopherol concentrations in the liver, which could be attributed to a higher demand for antioxidants and mobilization of α-tocopherol from tissues to the blood [12, 23].

The results of the present study confirmed that α-tocopherol prevents lipid peroxidation. Vitamin E plays a very important role in reducing exercise-induced lipid oxidation, and the results of sever-

Table 5. Correlations among the variables (r)

	Concentration of α-tocopherol in liver	**TBARS**	**Hepatic 8-OHdG**
Doses of α-tocopherol	0.498 p = 0.001	–0.571 p < 0.0001	–0.180 p = 0.271
Training	–0.521 p = 0.001	0.451 p = 0.004	0.132 p = 0.421
Concentration of α-tocopherol in liver	_	–0.609 p < 0.0001	–0.529 p < 0.0001

al studies involving humans and animals indicate that the administration of α-tocopherol reduces lipid peroxidation [24–26]. Kinoshita and Tsuji [27] evaluated the effects of strenuous exercise and vitamin E supplementation on lipid peroxidation in rat livers. In their study, the highest concentrations of TBARS were found in animals not receiving vitamin E, and the lowest in rats that were undergoing training while on a diet supplemented with vitamin E.

Treadmill running causes stress and mechanical injury, and electrical stimulation also induces ROS generation. In the present experiment, the training produced higher levels of oxidative stress by increasing lipid peroxidation and lowering α-tocopherol concentrations in the liver. Hepatic ischemia-reperfusion injuries increased ROS formation and promoted adenosine triphosphate (ATP) depletion in the liver. The administration of α-tocopherol accelerated ATP recovery and strengthened the antioxidant defense system of hepatocytes [28].

Hepatocytes are not indifferent to the genotoxic effect of physical activity, which could lead to genetic instability [29]. In this study, no changes in 8-OHdG levels were observed in the liver. This could be attributed to the intensity of exercise, which is more likely to determine the extent of DNA damage than the duration of exercise. Moderate and/or intense aerobic exercise is not sufficient to induce a significant increase in 8-OHdG levels, probably due to accelerated DNA repair [30]. Research suggests that regular exercise, such as the type used in the present study, could contribute to adaptation to oxidative stress, which could explain the observed lack of differences in 8-OHdG levels [7, 27, 31]. Similar results were reported by Kinoshita and Tsuji [27] and by Wawrzyniak et al. [16], who demonstrated that endurance exercise had little effect on hepatic DNA damage and that 8-OHdG levels did not differ significantly between training and non-training subjects. Pozzi et al. reported significantly higher levels of DNA damage in hepatic tissue after strenuous exercise [29]. According to Radak et al., exercise training accelerates DNA repair [31]. In studies by Nakamoto et al. [32] and Ohkuwa et al. [33], endurance training decreased 8-OHdG levels in rat livers. In contrast, Ogonovszky et al. [7] found that 8-OHdG levels in the liver did not change after moderate training, but increased in the overtrained group. In the current experiment, the rats underwent endurance training, which seems insufficient either to induce DNA damage or to protect DNA against free radicals more effectively than lipids or proteins [26]. In spite of this, the strong negative correlations between α-tocopherol levels and the concentrations of TBARS and 8-OHdG in the liver emphasize the importance of hepatic α-tocopherol levels, which are affected by α-tocopherol intake.

The results of the present study indicate that adequate dietary intake of α-tocopherol could protect previously untrained young subjects against oxidative damage induced by intensive exercise. According to Llorente-Cantarero et al., "oxidative tissue damage might have more serious consequences in children than in adults" [34]. The specific markers of oxidative stress and their role in pediatric diseases have not yet been identified.

This study has demonstrated that endurance training causes lipid peroxidation and reduces the concentration of α-tocopherol in the liver, but does not contribute to DNA damage. Alpha-tocopherol had a protective effect on lipids and DNA in the livers of young animals subjected to endurance training. The results of this study suggest that adequate intake of α-tocopherol could provide health benefits for previously untrained young subjects. These findings may be useful in the process of formulating nutritional recommendations for children commencing physical training.

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