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Serum Ex Vivo Lipoprotein Oxidizability in Patients with Ischemic Heart Disease Supplemented with Vitamin E

A. NAGYOVÁ, V. MONGIELLOVÁ, Z. KRIVOŠÍKOVÁ, P. BLAŽÍČEK[1], V. SPUSTOVÁ, M. GAJDOŠ, R. DZÚRIK

Institute of Preventive and Clinical Medicine, Bratislava, Slovak Republic, [1]Military Hospital, Bratislava, Slovak Republic

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Summary

The decreased oxidizability of plasma lipoproteins is related to the increased vitamin E intake and its association with a relatively lower incidence of coronary heart disease has been proposed. We investigated the effect of the *in vivo* vitamin E supplementation on the oxidizability of serum lipids in patients with ischemic heart disease and a moderate hypercholesterolemia. Thirty-two patients (16 males and 16 postmenopausal women) participated in this placebo-controlled, randomized trial. They were treated with 400 mg vitamin E/day for 6 weeks. The copper-induced serum lipid oxidizability *ex vivo* was assessed by measuring conjugated diene formation at 245 nm. We also measured vitamin E, malondialdehyde (MDA) and uric acid concentrations in the plasma. Because of observed significant differences in parameters of serum lipid oxidizability (lag time and maximal rate of oxidation), plasma α-tocopherol and MDA levels between male patients and postmenopausal women supplemented with vitamin E, the results were compared between both genders. Six weeks of vitamin E supplementation significantly increased plasma vitamin E levels (by 87 %) in male patients but in postmenopausal women only by 34 %. Concomitantly with increased plasma levels of vitamin E the decrease in plasma MDA levels was observed in male patients (decrease by 20 %; p=0.008), but in postmenopausal women the decrease did not attain statistical significance. Plasma uric acid levels were not apparently changed in placebo or vitamin E supplemented groups of patients. The changes in *ex vivo* serum lipid oxidizability after vitamin E, supplementation have shown a significantly prolonged lag time (by 11 %; p=0.048) and lowered rate of lipid oxidation (by 21 %; p=0.004) in male patients in comparison with postmenopausal women. Linear regression analysis revealed a significant correlation between plasma vitamin E levels and the lag time (r=0.77; p=0.03) and the maximal rate of serum lipid oxidation (r=-0.70; p=0.05) in male patients. However, in postmenopausal women the correlations were not significant. We conclude that 400 mg vitamin E/day supplementation in patients with ischemic heart disease and a moderate hypercholesterolemia influenced favorably *ex vivo* serum lipid oxidation of male patients when compared with postmenopausal women. The observed differences between both genders could be useful in the selection of the effective vitamin E doses in the prevention of coronary heart disease.

Key words

Serum lipoprotein oxidizability • Vitamin E • Coronary heart disease

Introduction

The increasing evidence implicates the oxidative modification of low density lipoprotein (LDL) as an important event contributing to atherogenesis. Simultaneously, a significant attention has been paid to the prevention of lipoprotein oxidation by antioxidants. The majority of studies have focused on the antioxidative effect of α-tocopherol, the most active form of vitamin E, which is the major lipid-soluble antioxidant in human LDL. Significant correlations were observed between the resistance of LDL to oxidation, vitamin E levels (Suzukawa *et al.* 1995) and the degree of coronary atherosclerosis (Regnström *et al.* 1992). Similarly, inverse correlations have been found in several epidemiological studies (Stampfer *et al.* 1993, Rimm *et al.* 1993, Gey *et al.* 1993) between plasma antioxidant vitamin levels (vitamin C, vitamin E and ß-carotene) and the mortality from coronary heart disease. However, the results of large clinical intervention trials are conflicting. In the Cambrige Heart Antioxidant Study (CHAOS) (Stephens *et al.* 1996), the risk of death or nonfatal myocardial infarction in patients with established coronary heart disease on a daily dose of 400 or 800 IU of vitamin E was significantly reduced, while the results of further large randomized trials have not proved the protective effect of vitamin E on cardiovascular disease, total mortality or incidence of fatal and nonfatal myocardial infarction (The ATBC Study 1994, The GISSI Study 1999, Yusuf *et al.* 2000). The chemical, biological and clinical aspects of vitamin E in atherogenesis, its antioxidative and prooxidative effects were recently reviewed by Neuzil *et al.* (2001).

The most frequent method used for determining the resistance of LDL to oxidation is to measure the kinetics of conjugated diene formation after *in vitro* copper-mediated oxidation (Esterbauer *et al.* 1989). The method of Regnström *et al.* (1993), where the lipid resistance to oxidation was measured in whole plasma or serum has been later used in several studies (Nyyssönen *et al.* 1997, Schnitzer *et al.* 1998, Spranger *et al.* 1998). Plasma or serum contain several hydrophilic constituents with an antioxidant activity such as ascorbic acid, urate, ceruloplasmin, albumin etc. protecting plasma lipoproteins against *in vitro* oxidation, which are removed during the LDL isolation. Serum urate and ascorbic acid were the strongest determinants of serum lipid resistance to oxidation both in Finnish smokers and non-smokers (Nyyssönen *et al.* 1997).

The role of vitamin E in coronary heart disease prevention in most studies of human subjects is based on plasma vitamin E status. Recently, the optimal plasma vitamin E level protecting against cardiovascular and cancer diseases has been suggested to be ≥30 μmol/l in combination with plasma vitamin C levels ≥50 μmol/l. Plasma ratios of vitamin C / vitamin E lower than 1.3-1.5 could be associated with an increased risk of cardiovascular disease, and this is consistent with the role of these two co-antioxidants, which together protect organism against deleterious attack of free radicals (Gey 1998).

In this study, we were interested whether vitamin E supplementation in patients with ischemic heart disease can also reduce *ex vivo* oxidizability of lipoproteins in the serum, where they can be protected by different hydrophilic constituents with antioxidant activity. In addition, we determined plasma malondialdehyde (MDA) levels as a marker of oxidative stress. Because of the different effects of vitamin E on serum lipid oxidizability parameters (lag time and maximal rate of oxidation), plasma α-tocopherol and MDA levels in male patients and postmenopausal women supplemented with vitamin E, the results were compared between both genders.

Methods

Thirty-two patients with ischemic heart disease and moderate hypercholesterolemia (16 men aged 37-72 years and 16 postmenopausal women aged 45-75 years) participated in this placebo controlled, randomized trial to compare the effect of vitamin E on serum *ex vivo* lipid oxidizability. All of the subjects were non-smokers. The patients used medication for the treatment of ischemic heart disease, but they did not take any other medication, vitamin or antioxidant dietary supplements. The exclusion criteria also included hormone replacement therapy, thyroid disease, diabetes mellitus and liver or renal disease. Patients ingested 400 mg vitamin E/day in the form of D,L-α-tocopherol acetate (Vitamin E cps, Slovakofarma, Hlohovec, Slovakia) in one dose after breakfast for 6 weeks. Patients of placebo group ingested a capsule once daily prepared for this purpose by the same manufacturer. All patients were advised not to change their low fat and low cholesterol diet for the duration of the study. Informed consent was obtained from all subjects and the study was approved by the Institute`s Ethics Committee.

Blood samples were collected into EDTA-containing vacutainer tubes after an overnight fast prior to the beginning of the experiment and after supplementation of vitamin E for 6 weeks. Uric acid concentration, total cholesterol, HDL cholesterol and triacylglycerols were determined enzymatically (Autoanalyzer VITROS 250). LDL cholesterol was calculated from the Friedewald formula. Vitamin E (α-tocopherol) was measured by high-performance liquid chromatography as previously described by Arnaud *et al.* (1991). Briefly, 0.2 ml serum was treated with 0.1 ml of ethanol containing an internal standard, and mixed. 0.5 ml of hexane was then added. The hexane layer was removed and evaporated to dryness under a stream of nitrogen. The solid residue was redissolved in ethanol and α-tocopherol separated and quantified by reverse-phase HPLC with reference to standards of known concentrations. Mean values of plasma lipid standardized α-tocopherol are expressed as μmol of α-tocopherol to mmol of total cholesterol plus triacylglycerols. MDA levels in the plasma were measured by high-performance liquid chromatography according to the method of Wong *et al.* (1987).

The oxidizability of serum lipids was measured in the fresh serum according to the method of Regnström *et al.* (1993) modified by Schnitzer *et al.* (1998). Serum was diluted 50-fold in 0.02 mol/l phosphate buffered saline (PBS) pH=7.4 containing 720 μM sodium citrate and 100 μM copper. Cu^{2+}-induced oxidation of serum lipids was monitored at 37 °C by continuous recording of absorbance at 245 nm every 3 min for 4 hours using a Beckman spectrophotometer DU-650 until there was no further increase in the formation of conjugated dienes. The serum oxidation lag time and the maximal rate of oxidation (V_{max}) were calculated from the oxidation curve. V_{max} was expressed as mabs/min. Statistical evaluation was performed using paired Student's t-test and linear regression analysis. Value of $p<0.05$ was taken as the criterion of significance.

Table 1. Plasma lipid, α-tocopherol and malondialdehyde levels and parameters of serum lipoprotein oxidizability in male patients supplemented with vitamin E.

	Placebo group (n=8)			Vitamin E group (n=8)		
	Before	**After**	**p**	**Before**	**After**	**p**
Age [years]	55 ± 11	55 ± 11	NS	62 ± 8	62 ± 8	NS
Body mass index [kg/m^2]	27 ± 3	26 ± 3	NS	27 ± 3	25 ± 3	NS
Blood pressure [mmHg]	136/87	132/86	NS	141/84	136/84	NS
Total cholesterol [mmol/l]	6.51 ± 0.68	6.56 ± 1.01	NS	6.46 ± 0.77	6.97 ± 0.77	0.024
HDL cholesterol [mmol/l]	1.30 ± 0.28	1.45 ± 0.27	NS	1.45 ± 0.54	1.36 ± 0.49	NS
LDL cholesterol [mmol/l]	4.15 ± 0.48	4.16 ± 0.74	NS	4.02 ± 0.71	4.95 ± 1.31	0.047
Triacylglycerols [mmol/l]	2.35 ± 1.07	2.13 ± 0.92	NS	2.20 ± 1.06	2.30 ± 1.57	NS
α-Tocopherol [μmol/l]	37 ± 11	38 ± 6	NS	32 ± 7	60 ± 21	0.009
Lipid standardized α-Tocopherol [μmol/mmol]	4.12 ± 1.03	4.48 ± 0.66	NS	3.72 ± 0.61	6.54 ± 1.97	0.002
Malondialdehyde [μmol/l]	1.72 ± 0.51	1.50 ± 0.28	NS	1.89 ± 0.48	1.51 ± 0.36	0.008
Uric acid [μmol/l]	396 ± 67.9	407 ± 71.1	NS	379 ± 66.4	379 ± 71.1	NS
Lag time [min]	71 ± 15	71 ± 14	NS	74 ± 11	82 ± 8	0.048
Maximal rate of oxidation [mabs/min]	6.5 ± 1.2	6.7 ± 1.0	NS	6.6 ± 1.7	5.2 ± 1.3	0.004
Absorbance at zero time	0.94 ± 0.07	0.95 ± 0.07	NS	0.92 ± 0.05	0.94 ± 0.09	NS

Values are means ± S.D. NS – not significant.

Results

No significant differences in lipid plasma levels of either males (Table 1) or postmenopausal women (Table 2) were observed in the placebo groups. However, vitamin E supplementation increased plasma levels of total cholesterol and LDL cholesterol in male patients by 8 % and 23 %, respectively. On the other hand, vitamin E supplementation did not affect the plasma lipid profile in women. It should be noted that all patients had total and

LDL cholesterol levels above the range of physiological values before and after the supplementation.

Plasma vitamin E levels increased significantly after 6 weeks of supplementation by 87 % in male patients and by 34 % in postmenopausal women. Plasma vitamin E levels in the placebo group did not change in male patients, while the levels of vitamin E increased significantly (p=0.014) in postmenopausal women. Concomitantly, with increased plasma vitamin E levels in the group of male patients after supplementation with vitamin E, a significant decrease in plasma MDA levels was observed (decrease by 20 %, p=0.008) (Table 1). In the group of postmenopausal women, a decrease in plasma MDA levels was also observed, but it did not attain statistical significance (Table 2). The changes in plasma uric acid concentrations, which is an effective hydrophilic plasma antioxidant, were not apparent either in placebo or in the vitamin E supplemented groups and were significantly higher in male than in female patients.

Table 2. Plasma lipid, α-tocopherol and malondialdehyde levels and parameters of serum lipoprotein oxidizability in postmenopausal women supplemented with vitamin E.

	Placebo group (n=8)			**Vitamin E group (n=8)**		
	Before	**After**	**p**	**Before**	**After**	**p**
Age [years]	59 ± 9	59 ± 9	NS	63 ± 9	63 ± 9	NS
Body mass index [kg/m^2]	27 ±4	26 ± 3	NS	25 ±4	26 ± 4	NS
Blood pressure [mmHg]	139/88	138/83	NS	136/84	135/84	NS
Total cholesterol [mmol/l]	6.84 ± 0.93	6.74 ± 0.59	NS	6.44 ± 1.41	6.58 ± 1.32	NS
HDL cholesterol [mmol/l]	1.44 ± 0.31	1.46 ± 0.43	NS	1.48 ± 0.42	1.40 ± 0.60	NS
LDL cholesterol [mmol/l]	4.59 ± 0.86	4.91 ± 1.06	NS	4.23 ± 1.42	4.40 ± 1.30	NS
Triacylglycerols [mmol/l]	1.79 ± 0.70	1.68 ± 0.53	NS	1.62 ± 0.76	1.75 ± 0.51	NS
α-Tocopherol [µmol/l]	31 ± 9	40 ± 10	0.014	35 ± 10	48 ± 15	0.031
Lipid standardized α-Tocopherol [µmol/mmol]	3.60 ± 0.88	4.75 ± 1.12	0.038	4.41 ± 1.05	5.76 ± 1.66	NS
Malondialdehyde [µmol/]	1.90 ± 0.54	1.56 ± 0.28	NS	1.69 ± 0.63	1.48 ± 0.32	NS
Uric acid [µmol/l]	267 ± 93	261 ± 72	NS	272 ± 53	258 ± 40	NS
Lag time [min]	63 ± 5	64 ± 6	NS	72 ± 6	69 ± 6	NS
Maximal rate of oxidation [mabs/min]	7.9 ± 1.2	7.4 ± 0.7	NS	6.5 ± 1.5	6.3 ± 1.0	NS
Absorbance at zero time	0.92 ± 0.05	0.90 ± 0.03	NS	0.92 ± 0.06	0.96 ± 0.11	NS

Values are means ± S.D. NS – not significant.

The changes in oxidizability of serum lipids, as measured by the lag time and maximal rate of copper-induced lipid oxidation (V_{max}), have shown a significantly prolonged lag time (by 11 %, p=0.048) and decreased rate of oxidation (by 21 %, p=0.004) in the male patients supplemented with vitamin E (Table 1). However, practically no changes in lag time and V_{max} after vitamin E supplementation were observed in women (Table 2). There were no significant differences in all serum oxidation parameters before and after placebo treatment in both placebo groups. The six-week vitamin E supplementation did not significantly affect the initial absorbance at 245 nm at the start of lipid oxidation in the serum, which can reflect an increased level of preformed conjugated dienes and the level of oxidation, respectively. The correlations between plasma vitamin E levels and parameters of serum lipid oxidizability in male patients and postmenopausal women before and after vitamin E supplementation are presented in Figure 1. Significant correlations between vitamin E levels, lag time and V_{max} were found in male patients supplemented with vitamin E while the same correlations were insignificant in postmenopausal women. All correlations between plasma vitamin E and MDA levels in placebo and vitamin E supplemented group of male and female patients had inverse trends but were not significant (correlation

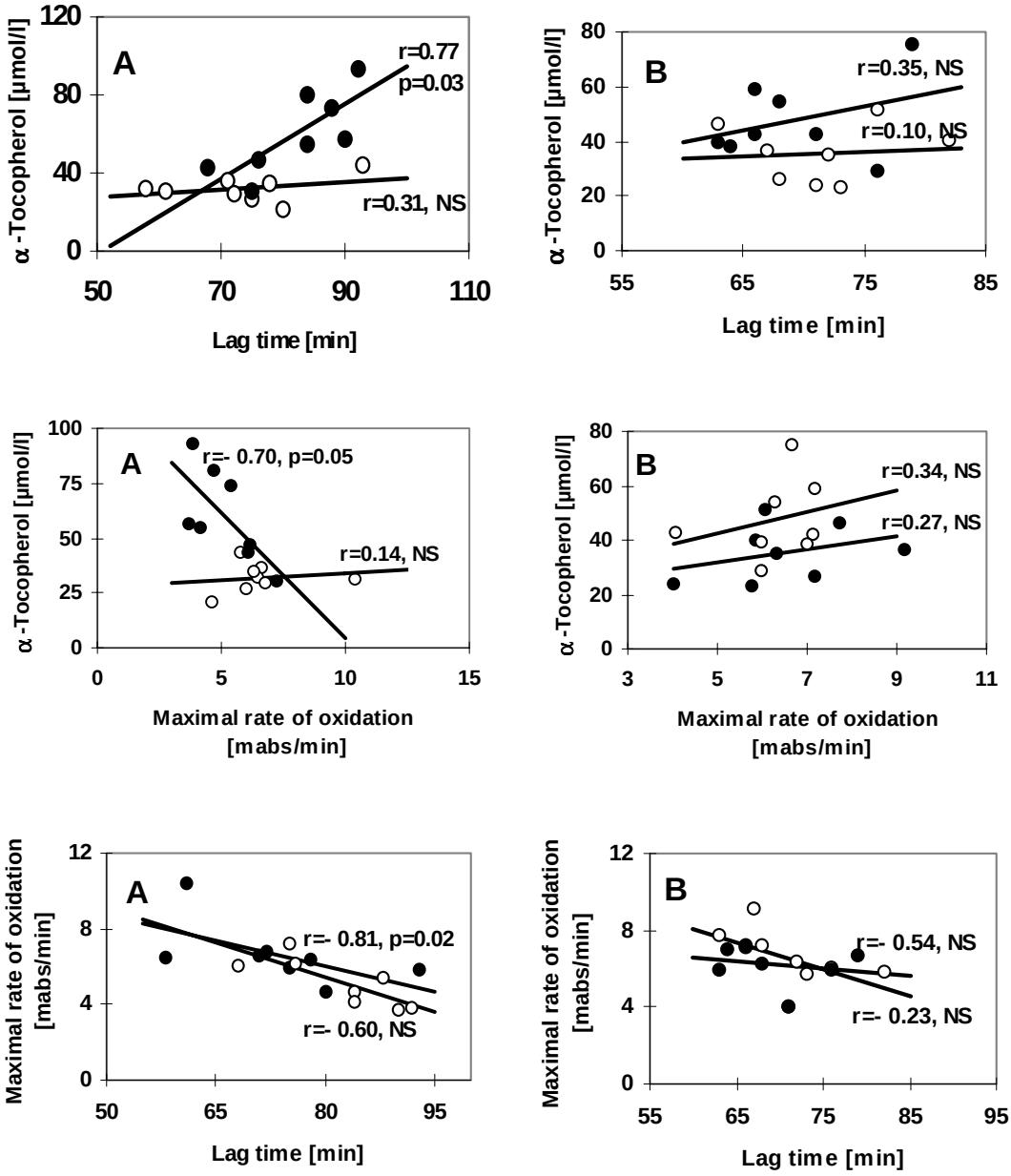

Fig. 1. *Relationship between plasma vitamin E levels and lag time and maximal rate of serum lipid oxidation in male patients [**A**] and postmenopausal women [**B**] before (o-o) and after (•-•) supplementation with 400 mg/day of vitamin E.*

coefficients from –0.11 to –0.56). Plasma vitamin E levels also correlated positively (but not significantly) with plasma uric acid concentrations in all groups of patients. As could have been expected, a significant positive association between plasma levels of uric acid and triacylglycerols and uric acid and lag time and consequently negative correlation between uric acid and the maximal rate of lipid oxidation were observed in placebo and vitamin E supplemented groups.

Discussion

The present study demonstrates that daily supplementation with 400 mg of vitamin E for 6 weeks reduces the oxidizability of serum lipoprotein in male patients with ischemic heart disease. However, the supplementation does not influence the *ex vivo* oxidation of serum lipoproteins in postmenopausal women. Vitamin E (α-tocopherol) is a highly efficient lipid

soluble antioxidant present in LDL increasing oxidation resistance of atherogenic plasma lipoproteins. Its inhibitory effect on plasma lipid oxidation was also observed by Spranger *et al.* (1998), who found a significant negative correlation between maximal rate of oxidation and vitamin E levels in plasma from healthy normolipidemic donors. In contrast to this, no correlation between plasma vitamin E content and the resistance of serum lipids to oxidation (lag time and maximal rate of oxidation) was observed in a population sample of Finnish smokers and non-smokers (Nyyssönen *et al.* 1997). In the present study, significant correlations between vitamin E levels, lag time and maximal rate of oxidation were found in male patients after vitamin E supplementation, but not before vitamin E supplementation. Similarly those correlations were insignificant in all groups of postmenopausal women. Therefore, our results are paradoxically comparable with the results of the above mentioned authors. Our results also indicate that plasma vitamin E levels are significantly higher after supplementation in male patients (increase by 87 %) while a relatively lower increase was observed in postmenopausal women (by 34 %). Vitamin E levels reached in the plasma of postmenopausal women are probably not sufficient to produce a significant effect on the *ex vivo* serum lipoprotein oxidation as in male patients. It should be noted that plasma vitamin E levels in all groups of patients before placebo or vitamin E administration were ≥ 30 μmol/l. The values ≥ 30 μmol/l have been suggested as the optimal plasma vitamin E levels, which can in combination with plasma vitamin C levels protect against cardiovascular and cancer diseases (Gey 1998). The long-term educational process of our patients to consume a nutritionally balanced diet could be, among others, the reason for the relatively normal baseline plasma vitamin E levels when compared with the lower plasma levels found previously in some selected groups of our population (Nagyová *et al.* 1998).

In the coincidence with the lower vitamin E increase in the plasma of postmenopausal women after supplementation we did not detect significant changes in plasma MDA levels, a known end product of lipoperoxidation. On the other hand, plasma MDA levels were significantly decreased after vitamin E supplementation in male patients. There are conflicting results in the literature concerning lipoperoxidation and antioxidant system in serum of patients with coronary artery disease (CAD). In some studies, plasma MDA levels were elevated in patients with CAD (Khan and Baseer 2000) while in others no increase in plasma MDA levels was observed. Consequently, the presence of variations in oxidant-antioxidant balance of serum of patients with CAD was suggested by Dogru-Abbasoglu *et al.* (1999) who observed a significant increase of the diene conjugate but not MDA levels in the serum of patients with CAD. Several methods are used by different research groups for the determination of plasma MDA. Therefore, it is difficult to compare MDA levels determined in our patients with the results of other studies. If they are higher than in healthy individuals or not, our results clearly indicate the beneficial effect of vitamin E supplementation on plasma MDA levels in the group of male patients, which decreased significantly by 20 % ($p=0.008$). These observations are strengthened by the fact that all correlations between plasma vitamin E and MDA levels in placebo and vitamin E supplemented groups of male and female patients, had a negative trend (correlation coefficients from – 0.11 to – 0.56). In the case of a higher number of patients in the groups, more relevant correlations could probably be expected.

Uric acid is an important scavenger of both hydroxyl and peroxyl radicals. According to Wayner *et al.* (1987) uric acid contributes 35-65 % to plasma total peroxyl radical trapping capacity (TRAP). According to Nyyssönen *et al.* (1997), serum urate and ascorbic acid concentrations were the strongest determinants of serum lipid oxidation resistance, expressed as lag time. In agreement with these observations we found a significant positive association between plasma uric acid levels and lag time (p values from 0.02 to 0.05) and negative correlations between uric acid and maximal rate of serum lipid oxidation (p values from 0.03 to 0.05) in placebo groups of patients. In our previous study with subjects with Down's syndrome and control healthy individuals serum uric acid levels were also significantly associated with the increased resistance of serum lipids to oxidation expressed as lag time (Nagyová *et al.* 2000).

We conclude that despite the relatively small number of patients in the groups, vitamin E supplementation at a dose of 400 mg per day during six weeks to the patients with ischemic heart disease and moderate hypercholesterolemia influences more favorably *ex vivo* lipid oxidation in the serum of male patients in comparison with postmenopausal women. The observed differences between genders could be useful in the selection of the effective vitamin E dosage in the prevention of coronary heart disease.

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Reprint requests

Anna Nagyová, Institute of Preventive and Clinical Medicine, Limbová 14, 833 01 Bratislava, Slovak Republic, e-mail: nagyova@upkm.sk