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Effects of Vitamin E Supplementation on Antioxidation and Lipid Profiles of Rats on Diets Supplemented with Cholesterol and Olive Oil*

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ABSTRACT : Lipid peroxidation (LPO) has been identified as an important component of atherosclerosis. In this study, the effects of supplementation with cholesterol (0.5%), olive oil (5%) and vitamin E (0.05%) on erythrocyte glutathione (GSH), plasma malondialdehyde (MDA), total cholesterol, HDL-LDL cholesterol and triacylglycerol, brain and liver MDA and GSH concentrations of rats were investigated. A total of 50 *Sprague-Dawley* male rats aged 6 months, and of equal body weight were used and fed a standard ration *ad libitum*. Animals were housed in the University of Selcuk, Veterinary Faculty Experimental Animals Unit. The experiment lasted 60 days and there were five experimental groups as follows: 1. Control, 2. Cholesterol (0.5%), 3. Olive oil (5%), 4. Cholesterol plus vitamin E (0.05%), 5. Olive oil plus vitamin E (0.05%). At the end of the experiment, blood samples were taken by cardiac puncture and erythrocyte GSH, plasma MDA, cholesterol, HDL-LDL cholesterol, triacylglycerol and also GSH and MDA concentrations in brain and liver tissue of rats were spectrophotometrically determined. Supplementation of olive oil and cholesterol into rat diets (groups 2 and 3) caused significant differences in lipid parameters; HDL cholesterol concentrations were increased in the olive oil group and LDL cholesterol was lower than in the cholesterol fed group. Moreover, these decreases in LDL and triacylglycerol concentrations were more significant with vitamin E supplementation. The high plasma MDA concentrations showed that lipid peroxidation occurred in the olive oil group and the highest brain MDA concentrations were determined also in the olive oil group. These findings suggest that vitamin E addition may decrease the sensitivities of several oils to oxidation and that monounsaturated fatty acids in olive oil may decrease the incidence of atherosclerosis by regulating blood lipid profiles. (**Key Words :** Vitamin E, Cholesterol, Olive Oil, MDA-GSH, Rat)

INTRODUCTION

The free radical-scavenging antioxidants play an important role in the *in vivo* defense systems against oxidative stress caused by active oxygen species. Lipid peroxidation causes oxidative damage to biological systems through free radical chain reaction in cellular and subcellular membranes (Lii et al., 1998). Therefore, supplementation of antioxidants may be beneficial to human health by decreasing exposure to oxidative stress. Among several lipophilic antioxidants that are involved in protection of membrane lipids against peroxidation, α-tocopherol may be of particular importance (Wrona et al., 2004; Morel et al., 2006; Tsai et al., 2008). Alpha tocopherol can efficiently interact with alcoxyl and peroxyl radicals. As a result of such an interaction, the radicals are converted into alcohols and hydroperoxides, whereas α-tocopherol is oxidized to a chromanoxyl radical, a relatively nonreactive species which can be repaired by ascorbate (Ferre et al., 2001). Vitamin E has eight different stereoisomers, of which the α-tocopherol is known to have the greatest biological activity. α-tocopherol is the most abundant lipid soluble antioxidant *in vivo* and acts as an important inhibitor of lipid peroxidation in membrane systems (Ipek et al., 2007; Lin and Chang, 2006). It has been observed that α-tocopherol can scavenge peroxyl radicals, singlet oxygen and superoxide anion radicals (Lii et al., 1998). In addition to antioxidant vitamins, other antioxidants and antioxidant enzymes protect biological systems from oxidative stress. Glutathione (GSH) has been shown to protect lipids from peroxidation in cytosolic and particulate subcellular components of the liver. Previous

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Table 1. Dietary composition and group supplements in the study

Nutrient	%
Corn	20
Barley	10
Wheat	17
Wheat-husk	12
Soybean meal	18.5
Corn gluten	15
Meat-bone powder	3.6
Vegetable oil	3
DCP	0.3
Vitamin-mineral premix[1]	0.6
Analysis of nutrients	
Dry mater	92.07
Crude protein	20.07
ME (kcal/kg)[2]	2,945
Crude cellulose	8.37
Crude oil	4.10
Ca[2]	0.61
Total P[2]	0.75
Digestibilable P[2]	0.37
Supplements according to groups	
Cholesterol (group 2 and 4)	0.5
Olive oil (group 3 and 5)	5
Vitamin E (group 4 and 5)	0.05

[1] Per 1 kg vitamin-mineral premix contains: 12,000 IU vitamin A, 2,400 IU vitamin D_3, 20 mg vitamin E, 4 mg vitamin K_3, 3 mg vitamin B_1, 7 mg vitamin B_2, 25 mg niacin (vit B_3), 10 mg panthotenic acid (vit. B_5), 5 mg vitamin B_6, 15 μg vitamin B12, 50 μg biotin, 1 mg folic acid, 50 mg vitamin C, 100 mg Mn, 60 mg Fe, 60 mg Zn, 5 mg Cu, 2 mg I, 500 μg Co, 150 μg Se.

[2] Calculated.

studies (Lii et al., 1998; Wrona et al., 2004) have shown that GSH can inhibit lipid peroxidation through the following mechanisms: scavenging of free radicals by GSH-dependent proteins, scavenging of peroxyl radicals by GSH, maintenance of membrane protein thiols by GSH, protection by GSH and α-tocopherol of a glutathione S-transferase isoenzyme responsible for the composition of lipid hydroperoxides, and a GSH dependent protein that regenerates α-tocopherol from the α-tocopherol radical. Animals are continuously exposed to oxidative stress of endogenous origins and the cumulative effects of this stress appear to play a role in many of the diseases associated with aging. Saturated fatty acid (SFA) intake is the dietary factor most closely related to total cholesterol concentrations. Cholesterol itself, having an unsaturated double bond between C5 and C6, is susceptible to oxidation and to producing various oxysterols (Lu and Chiang, 2001). On the other hand, some research has also focused on the potential role of cholesterol in protecting lipid membranes against peroxidation; similar evidence has been further demonstrated in a variety of systems (Smith, 1991). According to several studies monounsaturated fatty acids (MUFAs) from olive oil, n-6 polyunsaturated fatty acids (PUFAs) mainly from corn oil and carbohydrates have a more or less equivalent hypocholesterolemic activity (Becker et al., 1983; Reaven et al., 1993).

The objective of this study was to determine the effects of supplementation of cholesterol (0.5%), olive oil (5%) and vitamin E (0.05%) on the erythrocyte GSH, plasma MDA, total cholesterol, HDL-LDL cholesterol and triacylglycerol concentrations, brain and liver MDA and GSH concentrations of 6 months-old, male Sprague-Dawley rats.

MATERIALS AND METHODS

Animals and husbandry

In this study, a total of 50 Sprague-Dawley male rats, aged 6 months and weighing approximately 299-350 g, were used. During the experiment, the rats received a standard laboratory diet with tap water *ad libitum*, except for an overnight fast before euthanasia. Animals were housed at the University of Selcuk, Faculty of Veterinary Medicine Experimental Animals Unit. Rats were weighed individually and divided into 5 main groups each of 10 rats and housed in standard polycarbonate cages (Tecniplast, Italy) under controlled humidity and temperature in a quiet room with 12/12-h light/dark cycles. Rats were clinically observed and weighed at 30 day intervals. The animals were handled according to the guidelines of the Faculty of Veterinary Medicine, University of Selcuk Ethical Committee and complied with the Care and the Use of Laboratory Animals. The experimental period lasted until the 60th day.

The composition of the basal diet is shown in Table 1. The basal diet was supplemented with olive oil (5%), cholesterol (0.5%) (powder Merck) and vitamin E (α-tocopherol, 0.05%). To avoid lipid peroxidation, diets were prepared weekly and were kept in dark plastic bags at 0°C.

Blood and tissue sample collection

At the end of the experimental period (60 days), rats were weighed and anaesthetised with ether. Blood samples were taken by cardiac puncture for RBC (erythrocyte) and plasma preparation and then rats were euthanized by the intracardial injection of formalin (10 ml). Brain and liver samples were collected, frozen in 10 ml sodium phosphate buffer (pH 7.4) and stored at -80°C until analysis. Plasma was obtained by centrifugation of blood at 1,500 g for 10 minutes at +4°C. Remaining RBC were washed five times with physiological saline (0.9% NaCl) and centrifugation at 1,500 g for 5 minutes at +4°C. Washed RBC (50 μl) were put into a test tube and distilled water (450 μl) and sulfosalisilic acid (10%, 500 μl) were added, stored in ice for 1 h and then centrifuged at 4,000 g for 3 minutes. The supernatant (200 μl) was used for erythrocyte GSH analysis (Elmann, 1959).

Table 2. Selected blood parameters obtained from the groups

Groups/parameters	Control		Cholesterol		Olive oil		Cholesterol plus vitamin E		Olive oil plus vitamin E	
	n	X±Sx	n	X±Sx	n	X±Sx	n	X±Sx	n	X±Sx
Plasma vitamin E (mg/dl)	9	0.446±0.03^{c}	9	0.451±0.02^{c}	9	0.473±0.04^{c}	9	0.797±0.03^{b}	10	0.961±0.05^{a}
Plasma MDA (nmol/ml)	9	4.08±0.08^{c}	9	4.45±0.09^{a,b}	9	4.67±0.17^{a}	9	4.52±0.12^{a,b}	10	4.30±0.08^{b,c}
Total cholesterol (mg/dl)	9	88.25±6.15^{b}	9	102.85±6.47^{a}	9	81.23±5.42^{b}	9	74.81±6.16^{c}	10	72.22±5.89^{c}
HDL cholesterol (mg/dl)	9	49.41±0.22	9	48.69±0.37	9	50.12±0.29	9	49.73±0.41	10	49.83±0.56
LDL cholesterol (mg/dl)	9	45.86±5.33^{b}	9	60.63±7.14^{a}	9	35.58±3.45^{b,c}	9	26.48±3.32^{c}	10	26.24±3.73^{c}
Triacylglycerol (mg/dl)	9	95.13±6.31^{a}	9	92.35±6.07^{a}	9	82.37±7.50^{a,b}	9	66.99±6.03^{b,c}	10	54.24±4.41^{c}
Erythrocyte GSH (μmol/g Hb)	9	5.36±0.24^{b}	9	4.80±0.16^{b}	9	7.13±0.48^{a}	9	5.34±0.40^{b}	10	5.53±0.38^{b}

$^{a-c}$ Means within lines with no common superscripts are significantly different (p<0.01), according to Duncan's multiple range tests.

Table 3. Selected tissue MDA and GSH levels obtained from the groups

Groups/parameters	Control		Cholesterol		Olive oil		Cholesterol plus vitamin E		Olive oil plus vitamin E	
	n	X±Sx	n	X±Sx	n	X±Sx	n	X±Sx	n	X±Sx
Liver MDA (nmol/g protein)	9	458.21±25.99	9	494.04±5.46	9	501.11±13.60	9	465.48±13.09	10	449.92±7.37
Brain MDA (nmol/g protein)	9	772.78±22.50^{b}	9	734.80±15.57^{b,c}	9	832.61±9.65^{a}	9	722.08±17.14^{c}	10	724.04±14.50^{b,c}
Liver GSH (nmol/g protein)	9	95.74±7.54^{b}	9	95.52±1.80^{b}	9	104.03±4.59^{b}	9	97.15±5.65^{b}	10	119.52±5.41^{a}
Brain GSH (nmol/g protein)	9	75.04±2.44^{b,c}	9	69.40±1.30^{c}	9	77.62±2.33^{a,b}	9	73.28±1.75^{b,c}	10	82.72±1.99^{a}

$^{a-c}$ Means within lines with no common superscripts are significantly different (p<0.01), according to Duncan's multiple range tests.

Analysis of plasma

Plasma vitamin E (α-tocopherol) concentrations were measured by a rapid spectrophotometric method of Martinek (1964). Briefly, plasma samples (1 ml) were saponified in the presence of 1 ml ethanol (absolute) and 1 ml xylene and then centrifugated at 1,500 g for 5 minutes. Supernatant (500 μl) was removed and mixed with 500 μl TPTZ (2,4,6 Tripiridil S-Triazin, Sigma) and 100 μl $FeCl_3$ and absorbance rapidly measured at 600 nm. LPO concentration was estimated spectrophotometrically as plasma MDA by the thiobarbutiric acid method (Draper and Hadley, 1990). Total plasma cholesterol, high density lipoprotein cholesterol (HDL), low density lipoprotein cholesterol (LDL) and triacylglycerol were determined with a spectrophotometer (Shimadzu, UV-2100 Japan) according to instruction manuals accompanying the diagnostic kits (Biosystem, GmBH).

Analysis of brain and liver tissue

Frozen tissues (described above) were weighed and prepared as homogenates (1.0 g tissue/10 ml 0.15 mol/L KCl) using a Ultrasonic Cell Disruptor (Misonix, Farmindale, NY). Homogenates were mixed with cool HClO4 (8%) and centrifuged at 3,000 g for 15 minutes at +4°C. The supernatant was used to determine MDA (Uchiama and Mihara, 1977) and GSH (Elmann, 1959) concentrations of brain and liver tissues.

Statistical analysis

All parameters obtained from the experimental groups were measured individually and analysed by one-way ANOVA. Any significant differences were further analysed by Duncan's multiple range test (SPSS, 1998).

RESULTS

The dietary protocol used in this study was effective, because it produced significantly different plasma vitamin E, total cholesterol and LDL cholesterol concentrations in an eight-week feeding period (Table 2). Supplementation of olive oil and cholesterol (groups 2 and 3) made significant differences in lipid parameters; also HDL cholesterol concentrations were increased (non significantly) in the olive oil group and LDL cholesterol was lower than in the cholesterol fed group (Table 2). Moreover, these decreases in LDL and triacylglycerol concentrations were more significant with vitamin E supplementation. The high plasma MDA concentrations showed that high lipid peroxidation occurred in the olive oil-added group and the highest brain MDA concentrations were also determined in the olive oil group. The highest erythrocyte and brain GSH concentrations (p<0.001) were obtained from olive oil and olive oil plus vitamin E groups, respectively, while the lowest brain MDA concentration was observed in the cholesterol plus vitamin E group (p<0.001, Table 3).

Table 4. Body weights (g) obtained from the groups

Groups	n	1 day	n	30 day	n	60 day
Control	10	298.85±9.38	10	324.60±11.68	9	341.62±9.40
Cholesterol	10	299.25±9.39	10	316.45±12.78	9	338.97±8.99
Olive oil	10	298.46±8.12	10	325.87±7.86	9	339.84±9.39
Cholesterol plus vitamin E	10	300.04±8.45	10	330.74±6.76	9	337.68±11.70
Olive oil plus vitamin E	10	299.37±10.34	10	333.02±5.54	10	352.31±7.42

DISCUSSION

Our results showed that a diet supplemented with cholesterol or olive oil plus vitamin E could lead to changes in blood lipid profile and oxidative stress systems in different tissues in the rat. As shown by changes in the serum lipid profile (Table 2), olive oil reduced hyperlipemia in our experimental model and we found a significant decrease in the LDL, total cholesterol and triacylglycerol concentrations especially in the olive oil plus vitamin E group. Previous studies have reported contradictory findings with regard to the effect of olive oil on plasma lipid profile. In experimental animals (Navaro et al., 1992; Shick et al., 1993) and in humans (Sirtori et al., 1992), it was found that a diet rich in olive oil led to no significant modifications in plasma lipids. In contrast, Grundy et al. (1988) and Wahrburg et al. (1992) showed that, in humans, olive oil reduced concentrations of LDL cholesterol and increased HDL cholesterol; these results were similar to the findings in the present study. The administration of olive oil-enriched diets was shown to induce the capacity of brain and liver tissues to produce lipid peroxides (Hammer and Wills, 1978; Navaro et al., 1992). However, research with the same experimental model as that used in the present study found that diets rich in n-6 fatty acids (such as virgin olive oil) modified oxidative stress in several tissues (De La Cruz et al., 1999). Clement and Bourre (1990) suggested that this effect of n-6 fatty acid derivatives might result from enhanced antioxidative defence mechanisms in the brain.

Our findings in animals given olive oil plus vitamin E are compatible with these results; when tissue oxidative stress increased, olive oil and vitamin E decreased lipid peroxidation and augmented the glutathione-based defence mechanism in erythrocyte, brain and liver tissues. Therefore, fish oil, PUFAs and olive oil-rich diets have to be adequately supplemented with antioxidants to avoid their adverse effects (Mattson and Grundy, 1985), but, compared with fish oil and PUFAs, olive oil shows a lower rate of oxidation and production of free radicals (Miret et al., 2003). Several mechanisms have been proposed to explain the antioxidant capacity of olive oil. One possible mechanism is the increase in tissue sensitivity to the lipid antioxidant effects of vitamin E (Scaccini et al., 1992). Another possibility, suggested by Reaven et al. (1993), is the antioxidant ability of oleic acid itself, the major component of olive oil. Recent work by De La Puerta et al. (1999) showed that the polyphenols in olive oil (oleuropein, tyrosol, hydroxytyrosol and caffeic acid) inhibit the *in vitro* activity of leucocyte 5 lipoxygenase, and also inhibit the formation of oxygen reactive species in these cells in a concentration-dependent way. These phenolic compounds present in virgin olive oil (that are lost during the refining process in a great extent) may increase the antioxidant capacity under high oxidative stress situations (Lokesh et al., 1981). However, we did not find reduced lipid peroxide (MDA) data in the olive oil group; the decreases shown in plasma and tissue MDA concentrations in the olive oil plus vitamin E group may be explained as the antioxidant effect of vitamin E which might have reduced the susceptibilities to peroxidation of monounsaturated fatty acids in olive oil. Increased number of double bonds in dietary PUFA results in increased susceptibility to lipid peroxidation, which could potentially contribute to the pathology of atherosclerosis. Ingestion of DHA or fish oil exhibited a greater increase in serum susceptibility to oxidative stress than did linoleic or oleic acid (olive oil) (Aguilera et al., 2003). Because olive oil has antioxidant capacities and long-chain PUFAs are more susceptible to oxidation (Reaven et al., 1993), these findings of Aguilera et al. (2003) are somewhat expected. Cholesterol intake always decreases liver GSH Px and NADP-linked dehydrogenase activities in rats, but controversial results exist in studies involving lipid peroxidation expressed as thiobarbutiric acid-reactive substances (TBARS) (Tsai et al., 1977; Mahfouz and Kummerow, 2000). Therefore, fish oil, PUFAs and olive oil-rich diets have to be adequately supplemented with antioxidants to avoid adverse effects as suggested in our study. Olive oil, the main source of the Mediterranean diet, which has a high concentration of beneficial, MUFA has the ability to lower serum total and LDL cholesterol without causing a decrease in HDL cholesterol concentrations (Mattson and Grundy, 1985; Katan et al., 1994; Etherson et al., 1999). Compared with fish oil and PUFAs, olive oil shows a lower rate of oxidation and production of free radicals (Miret et al., 2003). The effect of dietary fat on HDL cholesterol has been intensely studied, primarily because HDL cholesterol concentration is a strong inverse predictor of cardiovascular diseases, even stronger than LDL cholesterol concentration.

In general, it is accepted that cholesterol lowering oils also cause a decrease in HDL (Mensink and Katan, 1992). However, some studies have found that MUFAs have no effect on HDL cholesterol (Mattson and Grundy, 1985; Grundy and Denke, 1990; Gardner and Kraemer, 1995) whereas others have reported increases (Mata et al., 1992; Perez-Jimenez et al., 1995) as presented in our experimental study. The antioxidant defence system in animals consists of antioxidant proteins that can be obtained by endogenous synthesis or by dietary means (Lii et al., 1998). Recently, Rojas and co-workers (1996) reported that antioxidant systems in the guinea pig heart, including GSH status, antioxidant enzymes and non-enzymatic antioxidants are related to dietary vitamin E concentration. Vitamin E is a major lipid soluble chain-breaking antioxidant and strongly inhibits the propagation of lipid peroxidation (Cheseman et al., 1986; Morel et al., 2006; Sahin et al., 2006). It can provide protection of lipoproteins against *in vivo* peroxidation by decreasing free radical oxidative damage to lipids.

As suggested previously (Hodis et al., 1992), under supraphysiologic cholesterol concentrations the antioxidant protection by plasma vitamin E may be insufficient to scavenge radical processes within a large lipid substrate pool and cholesterol may become oxidized (Smith, 1996). Supplementation with antioxidant vitamin E may lead to a significant rise in plasma concentrations, thus preventing or minimizing cholesterol oxidation. MDA value is a good indicator of oxidative stress and other indices, such as activity of GSH, just reflects the pathological response of tissue to oxidative stress (Li et al., 2007). In the present study, no statistically significance in plasma MDA concentrations was found in cholesterol and cholesterol plus vitamin E groups, but, as an antioxidant, vitamin E had a significant importance ($p<0.01$) in decreasing plasma MDA concentrations of the olive oil group (Table 2). Antioxidant supplementation of a cholesterol-rich diet may protect VLDL from alteration and consequently decrease its atherogenic effect and increase its binding affinity to hepatic receptors, which may enhance its clearance from the circulation. Cholesterol feeding increases lipid peroxidation (Sulyok et al., 1984; Bulur et al., 1986) and indications of *in vivo* modification of VLDL were noted previously (Parthasarathy et al., 1989). LDL and VLDL from hypercholesterolemic rabbits were also found to be more susceptible to *in vitro* oxidation and had lower α-tocopherol concentrations than LDL from normolipidemic rabbits.

On the other hand, cholesterol has also been proposed to exhibit its protective effect against oxidative stress either by antioxidant action as a radical scavenger, or by membrane stabilization owing to a steric hindrance between two adjacent unsaturated acyl chains (Smith, 1996). Although cholesterol itself can be oxidized by various initiators or oxidants, it is relatively stable; for example, a higher radiation dose is required to oxidize the cholesterol molecule than that required for the oxidation of double bounds of phospholipids.

The lower amount of PUFAs as substrate might be more resistant to lipid peroxidation. As a result, the TBARS (MDA) of serum and liver in cholesterol-fed rats maintained lower values than in cholesterol-free rats (Lu and Chiang, 2001).

In our study, brain MDA concentrations in cholesterol-fed rats were lower than that of control and olive oil groups ($p<0.01$); however, plasma MDA values were unexpectedly higher ($p<0.01$) than in the control.

In conclusion, the present study shows that, despite its antiatherogenic properties, olive oil, commonly used in human nutrition, may increase lipid peroxidation in blood and several tissues of rats. Therefore olive oil-rich diets have to be adequately supplemented with antioxidants such as vitamin E to avoid oxidative effects. However, it is better to study the effects of different dosage of olive oil in the diet on some histological indexes of the cardiovascular system.

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