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Influence on Hazelnut Oil Administration on Peroxidation Status of Erythrocytes and Apolipoprotein B 100-Containing Lipoproteins in Rabbits Fed on a High Cholesterol Diet

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Hazelnut oil (HO) is rich in monounsaturated fatty acids (MUFA). The effect of a high cholesterol (HC) diet with and without HO on lipids and lipid peroxide levels in plasma, apolipoprotein B 100-containing lipoproteins (VLDL + LDL), and erythrocytes as well as hematological data was investigated in rabbits. A HC diet caused significant increases in lipid peroxide levels in plasma and apo B-containing lipoproteins together with histopathological atherosclerotic findings in aorta. In addition, this diet resulted in hemolytic anemia associated with increased endogenous diene conjugate (DC) levels, but H_2O_2-induced malondialdehyde (MDA) levels remained unchanged in erythrocytes. HO supplementation reduced lipid peroxide levels in plasma and apolipoprotein B 100-containing lipoproteins as well as aortic atherosclerotic lesions in rabbits fed an HC diet without any decreasing effect on lipid levels. In addition, HO was found to reduce hemolytic anemia together with significant decreases in DC and H_2O_2-induced MDA levels.

KEYWORDS: Hazelnut oil; high cholesterol diet; lipid peroxidation; erythrocytes; apo B-containing lipoproteins; rabbit

INTRODUCTION

Rabbits are susceptible to the development of atherosclerosis (*1*), and HC diets have been shown to result in alterations in prooxidant and antioxidant status in plasma (*2*, *3*), plasma lipoproteins (*4*−*6*), liver (*2*, *3*, *7*), and aorta (*3*, *6*, *7*) as well as typical atherosclerotic changes in the aorta (*3*, *6*, *7*). Erythrocyte lipid peroxidation also increased (*8*), and the antioxidant system was affected by a HC diet in rabbits (*2*). A HC diet has been found to cause hemolytic anemia in rabbits (*8*, *9*). An increase in oxidative stress has been suggested as a cause of hemolytic anemia, which was seen following the HC diet (*8*). Increased oxidative stress is also found in the erythrocytes of hypercholesterolemic subjects (*10*, *11*). However, some investigators have suggested that cholesterol may play an antioxidant role in biological membranes (*12*) and protects the erythrocytes from oxidative stress (*12*, *13*).

On the other hand, the composition and amount of food, especially fats, are effective factors on prooxidant−antioxidant balance in the organism (*14*). Besides cholesterol-enriched feeding, PUFAs have been shown to cause an increase in the susceptibility of the organism to lipid peroxidation (*15*−*17*). MUFAs have been reported to lower the susceptibility of the organism to lipid peroxidation (*7*, *18*) and LDL oxidation (*19*, *20*).

HO is rich in MUFAs (*21*). In addition, HO has been known to be rich in several antioxidants such as vitamin E, flavanoids, and luteolin (*22*). Seventy-five percent of HO production in the world is provided from Turkey, and the use of HO has increased in the last years (*23*). However, the effect of its consumption on serum lipids and lipid peroxides is not known. Therefore, in this study, we investigated the effects of HO on lipids and lipid peroxidation in plasma and apo B 100-containing lipoproteins (VLDL + LDL) and erythrocytes as well as hematological data in rabbits fed both normal and cholesterol-supplemented diets.

MATERIALS AND METHODS

Animals and Diets. Male New Zealand white rabbits weighing 2.0−2.5 kg were used for all of the experiments. Animals were obtained from Eczacıbaşı Pharmaceutical Company (İstanbul, Turkey). The animals were divided into four groups: (i) control group ($n = 6$): The animals were fed with commercial rabbit chow containing 11% moisture, 10% crude ash, 15% protein, 3.5% crude fat, 47% carbohydrate, 7.5% cellulose, 3.5% salt mixture (AIN 76), and 1% vitamin E mixture (AIN 76) (w/w). (ii) HO group ($n = 6$): Rabbits were fed with a control diet enriched with HO (w/w) (5% for 8 weeks, 7.5% for 4 weeks, and 10% for 2 weeks, successively). (iii) HC group ($n = 8$): The animals were fed with a control diet enriched with cholesterol (w/w) (0.5% for 8 weeks, 0.75% for 4 weeks, and 1% for 2 weeks, successively). (iv) HO + HC group ($n = 6$): The animals were fed a control diet enriched with cholesterol plus HO, respectively (0.5 + 5% for 8 weeks, 0.75 + 7.5% for 4 weeks, and 1 + 10% for 2 weeks, successively).

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Table 1. Effect of HO on Lipids, DC, and MDA Levels in Plasma, Apo B 100-Containing Lipoproteins (VLDL + LDL) and Erythrocytes as Well as Hematological Data in Rabbits Fed a HC Diet (Means ± SD)[a]

	control ($n = 6$)	HO ($n = 6$)	HC ($n = 8$)	HC + HO ($n = 6$)
		whole blood		
erythrocyte numbers (million/mm^3)	6.16 ± 0.27[a]	6.03 ± 0.30[a]	3.42 ± 0.49[b]	4.37 ± 0.46[c]
Hb (g/dL)	13.3 ± 0.92[a]	13.5 ± 0.89[a]	7.92 ± 0.86[b]	10.45 ± 0.74[c]
Hct (%)	37.0 ± 1.70[a]	36.7 ± 1.78[a]	23.4 ± 2.23[b]	27.9 ± 1.81[c]
reticulocyte number (%)	0.4 ± 0.15[a]	0.50 ± 0.32[a]	7.00 ± 1.60[b]	5.0 ± 1.41[c]
		plasma		
cholesterol (mmol/L)	3.15 ± 0.49[a]	2.62 ± 0.56[a]	38.6 ± 6.51[b]	45.4 ± 8.04[b]
triglyceride (mmol/L)	0.63 ± 0.21[a]	0.63 ± 0.07[a]	1.62 ± 0.37[b]	3.67 ± 1.26[c]
phospholipid (mmol/L)	1.54 ± 0.14[a]	1.75 ± 0.18[a]	14.9 ± 2.92[b]	16.4 ± 2.10[b]
DC (μmol/L)	116.2 ± 13.7[a]	117.5 ± 17.0[a]	269.7 ± 50.0[b]	189.5 ± 38.2[c]
MDA (μmol/L)	3.13 ± 0.28[a]	3.46 ± 0.49[a]	7.92 ± 0.87[b]	5.53 ± 0.62[c]
		VLDL + LDL		
cholesterol (mmol/L)	0.79 ± 0.17[a]	0.76 ± 0.16[a]	19.2 ± 2.85[b]	21.1 ± 1.95[b]
DC (μmol/L)	28.0 ± 1.78[a]	27.7 ± 3.44[a]	84.5 ± 18.1[b]	59.0 ± 14.7[c]
		erythrocytes		
cholesterol (mmol/L)	2.18 ± 0.17[a]	2.11 ± 0.16[a]	2.91 ± 0.34[b]	2.60 ± 0.24[bc]
phospholipid (mmol/L)	2.20 ± 0.28[a]	2.08 ± 0.19[a]	2.17 ± 0.19[a]	2.17 ± 0.18[a]
cholesterol:phospholipid (mol:mol)	1.00 ± 0.09[a]	1.01 ± 0.13[a]	1.34 ± 0.17[b]	1.19 ± 0.10[ab]
DC (nmol/mL erythrocyte)	240.5 ± 25.4[a]	252.8 ± 45.5[a]	315.1 ± 42.2[b]	215.5 ± 30.1[ac]
MDA (nmol/g Hb)	255.7 ± 22.3[a]	246.5 ± 31.4[a]	212.4 ± 32.5[a]	131.0 ± 30.1[b]

[a] HO, hazelnut oil group; HC, high cholesterol group; HC + HO diet, hazelnut oil + high cholesterol group. Values not sharing a common superscript letter are significantly different by ANOVA (THS) test, $p < 0.05$.

HO and cholesterol were supplied from Ordu Sanayii A.Ş (Turkey) and Sigma (U.S.A.), respectively. The diets were stored at 4 °C. The animals were allowed free access to food and water and were kept in wire-bottomed stainless steel cages. The fatty acid composition of the diets was measured in the laboratories of Unilever Company. According to this, control and HC (1%) diets contained approximately 0.8 g of SFA, 1.0 g of MUFA, and 1.8 g of PUFA per 100 g of diet. Furthermore, HO (10%) and HC + HO (1% + 10%) contained 1.1 g of SFA, 10.3 g of MUFA, and 2.2 g of PUFA per 100 g of diet. The experimental procedure used in this study met the guidelines of the Animal Care and Use Committee of the University of Istanbul.

Sample Collection and Preparation. At the end of the feeding period of 14 weeks, the animals were fasted overnight and they were anesthesized with sodium pentobarbital (50 mg/kg, i.p). Blood was collected in tubes containing ethylenediaminetetraacetic acid (EDTA) by cardiac puncture. Plasma samples were obtained by centrifugation, and they were stored at −70 °C until analysis.

Biochemical Analysis. The following determinations were made on whole blood: the erythrocyte and reticulocyte numbers and Hb and Htc values (*24*). Plasma cholesterol and triglyceride levels were measured with the enzymatic kits from Sigma. Phospholipid levels were determined in plasma lipid extracts. After oxidative digestion, liberated inorganic phosphorus was determined (*25*). The degree of endogenous lipid peroxidation in plasma was also assessed by two different methods. Fırst, MDA levels were determined according to the method of Buege and Aust (*26*). Second, DC formation was measured in plasma chloroform−methanol (2:1) extracts. Extracted lipids were evaporated and dissolved in cyclohexane, and the DC was measured at 233 nm (*26*).

Apo B 100-containing lipoproteins (VLDL + LDL) were precipitated by dextrane sulfate and $MgCl_2$ solution, pH 7.0. The pellet was redissolved with phosphate-buffered saline (0.68 M NaCl, 10 mM NaH_2P0_4, pH 7.0) (*27*). VLDL + LDL oxidation was estimated by the endogenous DC levels in lipid fraction. Lipids were extracted from VLDL + LDL samples by choloroform−methanol (2:1), dried under nitrogen, then redissolved in cyclohexane, and analyzed spectrophotometrically at 233 nm (*28*).

Lipids of the erythrocyte were extracted with 2-propanol−chloroform (11:7, v/v) by the method of Rose and Oklander (*29*). After the lipids were evaporated, erythrocyte lipids were redissolved in 2-propanol and cholesterol levels were assayed by the enzymatic kits provided from Sigma. Phospholipids (*25*) and DC (*26*) levels were measured in the lipid extracts of erythrocytes as described for the plasma. The susceptibility of erythrocytes to lipid peroxidation was measured by determining MDA production after a 2 h incubation period with H_2O_2 at 37 °C according to the method of Stocks and Dormandy (*30*). The final composition of the incubation mixture was 5 mM H_2O_2, 2 mM sodium azide, and erythrocyte suspension in phosphate-buffered saline (30 mg Hb/mL incubation mixture). Values were expressed as nanomoles of MDA per gram of Hb. Hb concentration of erythrocyte suspensions was measured by Drabkin's reagent (*24*).

Histopathological Examination. Aortas were dissected and fixed in 10% buffered formalin solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histological studies.

Statistical Analysis. The results were expressed as the mean ± SD. Statistical analysis was performed by a one way analysis of variance (ANOVA) followed by Tukey's honestly significant difference post-hoc test.

RESULTS

The weight gain in rabbits was not significantly different among groups fed HO, HC, and HC + HO diets during the 14 week period (data not shown). In addition, no allergic reactions were not observed during the experimental period.

The results are shown in **Table 1**. According to this, (i) the HC diet caused significant decreases in the number of erythrocytes and Hb and Htc values and an increase in reticulocyte numbers as compared to controls. Erythrocyte numbers and Hb and Hct levels increased, but reticulocyte number values decreased after the HC + HO diet as compared to the HC group.

(ii) The HC diet resulted in significant increases in plasma cholesterol, triglyceride, and phospholipid levels as well as plasma DC and MDA levels. Plasma cholesterol and phospholipid levels did not change, triglyceride levels increased, and MDA and DC levels decreased in the HC + HO group as compared with the HC group.

(iii) Cholesterol and DC levels increased in VLDL + LDL lipoproteins following the HC diet. Although cholesterol levels remained unchanged, DC levels were observed to be decreased in the HC + HO group as compared with the HC group.

(iv) The HC diet resulted in increases in erythrocyte cholesterol levels, but it did not affect erythrocyte phospholipid levels as compared to the controls. The cholesterol:phospholipid

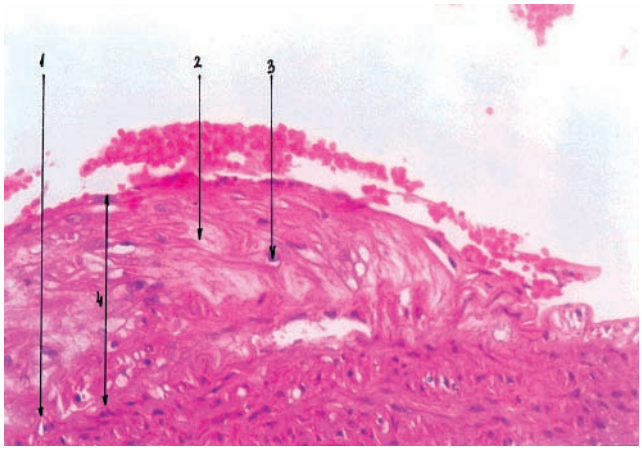

Figure 1. Histopathological findings of the aorta in the cholesterol-fed rabbits (magnification × 310, stained with hematoxylin–eosin); 1, internal elastic lamina; 2, foam cell; 3, macrophage; 4, atheromatous plaque.

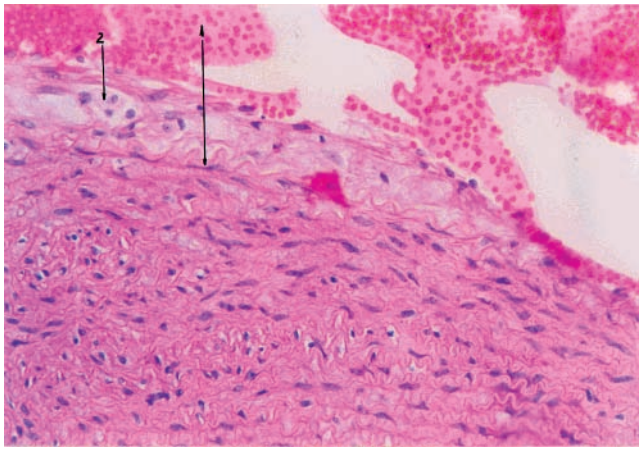

Figure 2. Histopathological findings for the aorta in the cholesterol plus HO-fed rabbits (magnification × 310, stained with hematoxylin–eosin); 1, internal elastic lamina; 2, macrophage.

ratio was elevated by the HC diet. Endogenous DC levels in erythrocytes were found to be increased, but H_2O_2-induced MDA levels remained unchanged in HC group. Erythrocyte cholesterol and phospholipid levels remained unchanged, but endogenous DC and H_2O_2-induced MDA levels were observed to be decreased following the HC + HO group as compared with the HC group.

(v) On the other hand, the HO diet did not have any effect on lipid and lipid peroxide levels in plasma, VLDL + LDL lipoproteins, and erythrocytes as well as hematological data in rabbits as compared to the control.

(vi) Aortic tissue from the HC group exhibited endothelial damage in some subendothelial areas, with foamy cell infiltration. The macrophage content in the cross-sectional lesioned area of the aorta was higher in the HC group, and typical atheromatous plaques were observed in the same area (**Figure 1**). Although atherom plaques formation was observed in the rabbits fed a HC diet, no such formation was observed in the rabbits fed HC + HO. However, a light-medium deposit of histiocytes was observed in the aortic intima in rabbits fed HC + HO (**Figure 2**). On the other hand, normal aortic structure was also seen in the HO group.

DISCUSSION

There is a continuous exchange of lipids between plasma lipoproteins and erythrocyte membranes, and severe hypercholesterolemia is known to lead to alterations in the erythrocyte lipid composition (*31*). Cholesterol-enriched erythrocytes have been reported to have several abnormal properties such as decreased membrane fluidity, increased hemolysis, decreased permeability to nonelectrolytes and electrolytes, and decreased activities of some membrane-bound enzymes (*31*, *32*). As it is known, free radicals and oxidative stress may cause changes in both membrane structure and function and these changes in turn may contribute to the erythrocyte hemolysis (*33*). Indeed, one of the mechanisms responsible for the changes of erythrocytes in hypercholesterolemia has been reported to be oxidative stress (*8*, *10*, *11*).

It has been reported that HO containing high amounts of vitamin E and flavoinoids has a higher total radical scavenger capacity and resistance to sunlight than olive oil (*34*, *35*). Although there is no study investigating the effect of HO on lipids and lipid peroxidation in serum and erythrocytes, Durak et al. (*36*) have shown that hazelnut supplementation caused decreases in serum cholesterol and MDA levels and an increase in serum antioxidant activity in healthy humans.

Erythrocytes are an appropriate model for lipid peroxidation studies (*33*). In our study, lipid peroxidation was evaluated by measuring both endogenous DC levels and H_2O_2-induced MDA levels in erythrocytes. We observed that the HC diet caused hemolytic anemia accompanied with increased endogenous DC levels in the erythrocytes of rabbits, but H_2O_2-induced MDA levels remained unchanged. The HC + HO diet did not alter plasma and erythrocyte cholesterol and phospholipid levels as compared to the HC group. This diet reduced the HC diet-induced hemolytic anemia in rabbits as well as endogenous DC levels and H_2O_2-induced MDA levels in erythrocytes. Therefore, the effect of HO on HC-induced hemolytic anemia may be related to its antioxidant power.

On the other hand, a few studies have examined the effect of a HC diet on LDL oxidation in rabbits (*4*−*6*). LDL is protected in vivo by antioxidants, and the susceptibility of LDL to oxidation depends on the balance between the prooxidants and antioxidants and the fatty acid composition. Copper-catalyzed LDL oxidation is most often used to evaluate the susceptibility of LDL to oxidation (*37*). Because the separation of LDL requires a time-consuming sequential ultracentrifugation and removal of EDTA by dialysis, these procedures may result in a partial degradation of apo B and reduce the vitamin E content of LDL (*27*, *28*). Therefore, faster isolation methods may be more suitable for the analysis of parameters of LDL oxidation. Ahotupa et al. (*28*) have described an assay based on determination of endogenous levels of conjugated dienes in lipids extracted from LDL. These authors have suggested that this assay is faster, simpler, and more applicable for clinical purposes. In cholesterol-fed animals, a large fraction of the plasma cholesterol present in lipoproteins are of VLDL but with β-electrophoretic mobility (*4*, *5*). Hornick et al. (*38*) have demostrated that VLDLs of rabbits contain apo B 100 and not apo B 48. Therefore, in our study, endogenous DC levels were determined in a VLDL + LDL fraction instead of LDL in rabbits fed an HC diet, and these levels have been found to be increased. Indeed, it has also been demostrated that LDL and β-VLDL from the hypercholesterolemic rabbits were more susceptible to oxidation than LDL from normolipidemic rabbits (*4*, *5*). In our study, HO has been found to cause a significant decrease in endogenous DC levels of apo B-containing lipoproteins in atherosclerotic rabbits but not in normal rabbits. Although similar results were obtained by olive oil rich in MUFAs (*19*, *20*), the results obtained with HO in this study are new.

Although it is not clear which mechanism plays a role in the reduction of the lipid peroxide levels in plasma, apo B-containing lipoproteins, and erythrocytes, the replacement of PUFAs by MUFAs in these tissue and/or antioxidant components found in the composition of HO seems to be effective.

ABBREVIATIONS USED

HC, high cholesterol; HO, hazelnut oil; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; MDA, malondialdehyde; DC, diene conjugate; VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; Hb, hemoglobin; Hct, hematocrit.

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