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Original investigation

LDL Oxidation and Vitamin E and Vitamin a Contents of LDL in Male Patients with Early-Onset Coronary Heart Disease and in Their 1st-Degree Male Relatives

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ABSTRACT

Objective: Coronary Heart Disease (CHD), of which the primary cause is atherosclerosis, is among the leading causes of mortality in the developed countries. Although many etiological agents have been considered responsible for the development of the disease, oxidized LDL (low-density lipoprotein) is the major risk factor. Formation of atherosclerotic lesion begins with the macrophages' turning oxidized LDL's into foam cells. Although many factors such as high LDL concentration, hypertension and low antioxidants in the system impair vascular structure and initiate this process, the present study is based on the genetic susceptibility and the risk groups.

The present study comprised the male patients with angiography-based diagnosis of early-onset coronary heart disease, as well as their sons or 1st-degree relatives, who are considered as high-risk group. It was aimed to compare these groups in terms of susceptibility of LDL to oxidation and to measure and compare vitamin E and A contents of LDL between these groups. Homocysteine concentration, which is considered as an independent risk factor for CHD, was also compared between the groups.

Materials and Methods: Susceptibility of LDL to oxidation was determined based on the formation of conjugated dienes observing for 200 minutes after being incubated with cupper (cu2+) ions. Vitamin E and A contents of LDL were measured by HPLC (High-performance Liquid Chromatography), which is considered as the reference method. Male patients with early-onset coronary heart disease (with >50% stenosis in at least two vessels on coronary angiography) and their 1st-degree relatives were compared in terms of above-mentioned three parameters. In addition, FPIA (Fluorescence Polarization Immuno-Assays) was used to measure homocysteine concentration.

Results: Three study groups were created: Group 1 consisted of 20 patients aged 40-55 years, who had >50% stenosis in 2 and/or more vessels detected on coronary angiography; Group 2 consisted of 18 subjects aged 18-20 years, who are the 1st-degree relatives (sons or brothers) of Group 1; Group 3 (control group) consisted of healthy males aged 18-35 years. Susceptibility of LDL to oxidation showed significant difference between Group 2 and Group 3. No statistically significant difference was determined between the groups in terms of LDL content of Vitamin A and E. Homocysteine concentration was significantly higher in Group 1 and Group 2.

Conclusions: Early-onset CHD can be prevented or delayed by means of relevant screening and treatment of high-risk groups in the population.

Keywords: LDL oxidation, early-onset coronary heart disease, vitamin E and A

INTRODUCTION

Coronary heart disease (CHD), the primary cause of which is atherosclerosis, is considered among the leading causes of mortality in the developed countries. It accounts for the great majority of all-cause mortality in some countries (2/3 of overall cardiac diseases, 1/3 of overall deaths). Atherosclerosis is the primary cause of CHD in the substantial proportion of these cases (1,4,18).

More emphasis has been put on the risk factors as this disease brings huge financial burden on the national economy. Many researches have been conducted concerning preventable risk factors (1,2,7,18).

In addition to the proven risk factors of atherosclerosis (hypercholesterolemia, smoking, hypertension, diabetes mellitus), the risk factors that could not be modified in anyway (non-modifiable) are considered as the permanent risk factors. Permanent risk factors include age, male gender, low socioeconomic level, and family history of early-onset CHD (5,19).

Comparing these non-modifiable risk factors, positive family history of early-onset CHD was determined via precise screening of the families with documented accumulation of personal risk factors. These risk factors have been defined to be the monogenic

factors such as phenotypic expressions and inheritance patterns and polygenic factors such as environmental conditions. Many earlier studies demonstrated that relevant risk factors are lacking in only 10% of such group of families but are responsible in 90%. Therefore, family members of the patients with CHD are the target population to assess the risk factors in early ages (1,2,19).

Human system develops antioxidant mechanisms against reactive products formed by endogenous and exogenous mechanisms. Numerous epidemiological studies have proven that atherosclerosis is closely associated with plasma proteins and fatty acids. Unsaturated fatty acids are more susceptible to oxidation. Many *invivo* and *invitro* studies demonstrated that LDL and VLDL, which undergo structural changes due to the oxidation of unsaturated fatty acid contents, play primary role in the initiation of atherosclerotic process. Free radicals produced in the absence of adequate antioxidant mechanisms, which are found in liquid media and in the structure of LDL and VLDL, lead to these chemical changes and play primary role in the formation of atherosclerotic lesions (1,3,4).

High homocysteine effect explained again by the mechanisms of free radical formation is the major risk factor for atherosclerosis (7,8).

In the light of above-mentioned information, patients with early-onset coronary heart disease with angiography-documented atherosclerotic vascular lesions were selected as the primary patient group.

As the second group, 18-35-year-old 1st-degree relatives (brothers or sons) of these patients were selected. Again, 18-35-year-old healthy males were selected as the control group.

It was aimed to determine the susceptibility of Apo B-containing lipoproteins (LDL, VLDL) to oxidation among the risk factors leading to atherosclerotic lesions, as well as to determine Vitamin A and E concentration in these lipoproteins and to assess homocysteine concentration, which is considered as the independent major risk factor for atherosclerosis, in the patients with early-onset CHD and in their 1st-degree relatives, who are at high-risk for CHD.

MATERIALS and METHODS

Patient Selection

A total of three study groups were created:

1. Group 1 (n=20): 40-55-year-old male patients with 50% stenosis in two and/or more vessels documented on coronary angiography, which was performed in the Cardiology Clinic in Dokuz Eylül University
2. Group 2 (n=20): 18-25-year-old 1st-degree male relatives (sons or brothers) of Group 1
3. Group 3 (n=18): 18-35-year-old healthy males

   All participants with diabetes mellitus, hypertension, hyperlipidemia and chronic kidney disease, smokers, those receiving antioxidants and lipid-lowering drugs were excluded from the study.

Biochemical analyses

Susceptibility of LDL to oxidation

Sample collection:

Venous blood sample drawn from the patients after 12-hour fasting period was added into the glass tubes containing EDTA with final concentration being 5mmol/L. The plasma was obtained after centrifuging the samples for 15 minutes at 800xg and +4^{0}C. The samples were analyzed in the same day without waiting.

Obtaining LDL:

Lipoproteins were obtained by the classical method (3): 500µL of the plasma was mixed with 50µ of 45g/L sodium phosphotungstate (pH 7.6) solution and 12.5 µL of 2mol/L $MgCl_2$ solution. It was centrifuged at 2000 Xg. After obtaining the supernatant, washing procedure was repeated for three times. Following the last centrifugation, the precipitant containing the lipoproteins was diluted again with 1ML of a buffer containing 10 mmol/L sodium phosphate and 1mol/L NaCl (pH 7.4) at 37^{0}C. Finally, 1 mL lipoprotein-containing study sample was obtained.

Detection of conjugated Diene (CD) to assess the susceptibility of LDL to oxidation:

Before starting oxidation, the protein in the samples was measured by Lowry Method (3,4), and the samples were diluted in the way protein concentration would be 0.1mg/mL. To induce oxidation, $CuSO_4$ $5H_2O$ was added into the 500µL of sample in the way final concentration would be 50µmol/L. The change in the absorbance of sample was monitored at 234nm for 200 minutes. Oxidation curve was drawn with x-axis representing time (minute) and y-axis representing absorbance for all samples. Duration of lag phase, progression phase and termination phase were identified on this curve.

Calculation of lag phase:

Lag phase on the oxidation curve is the area between the starting point and the point where oxidation gains acceleration. A tangential line is drawn from the acceleration point to the point where oxidation terminates and makes a plateau (a). Lag phase is calculated determining the intersection point of these lines and the time at that point.

Progression phase: the area between the acceleration point and the termination point where the curve is flattened (b).

Termination Phase:

The area where oxidation is terminated and the curve is flattened (c).

Maximum oxidation ratio:

Maximum oxidation ratio is calculated as the difference (Δabs) between the absorbance value of the intersection point of the lines b and c and the absorbance value of the intersection point of the lines a and b divided by the time difference between these points (Δt) and multiplying with oxidation constant (33.8), and maximum LDL oxidation ratio per protein is calculated dividing by the protein content of LDL (0.1) mg.

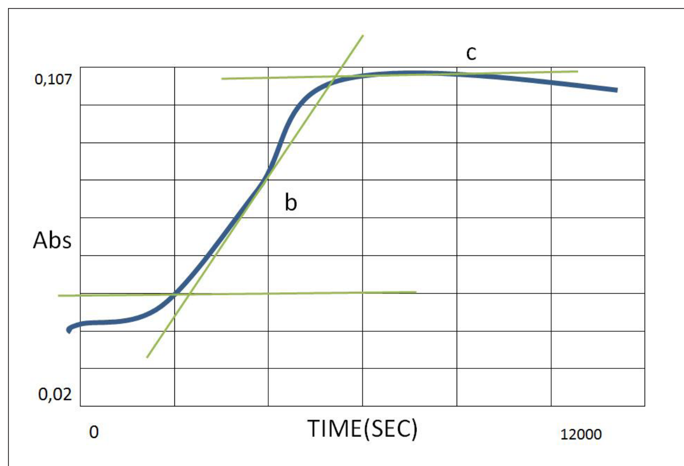

Figure 1. LDL oxidation curve

Maximum oxidation ratio = (ΔAbs/Δ(min).33.8 /0.1

Maximum CD concentration is calculated as the absorbance at maximum oxidation point minus initial absorbance divided by molar absorptivity coefficient (28000 L mol).

$$\text{Maximum CD concentration (mmol/mL)} = \frac{\text{maximum absorbance value- initial absorbance}}{28000}$$

The result was divided as the amount of sample was 500 microliters and the final result was presented in mmol/mL.

HPLC method was used to measure vitamin A and vitamin E contents of APO B-containing lipoproteins.

Following items were used for the analysis of following parameters:

Mobile phase compound:	70% acetonitrile
	20% dichloromethane
	10% methanol
Mobile phase flow rate:	1 mL/min for Vitamin E
	0.5mL/min for Vitamin A
Wavelength:	291nm for alpha tocopherol
	325nm for Beta-carotene
Standards:	2.5, 5, 10 and 20 micromole/mL for Vitamin A
	0.875, 1.75, 3.5 and 7.0 for vitamin E

Statistical Analysis

Statistical analyses were done by SPSS package program. Different statistics were used between different groups; student t-test was used for paired samples between Group 1 and Group 2, independent samples t-test was used between the controls and sons.

Graphic selection

Central tendency "Box-plot" graphic, where distribution and media are illustrated, was selected.

Group 1, Group 2, Control Group (Group3)

Table 1. Group 1 (Patient) LDL oxidation parameters

Group no	Lag Phase (minute)	Maximum oxidation ratio (μmol/mg protein LDL minute)	Maximum CD* concentration (nmol/mL)	Vitamin E (nmol/mg protein LDL)	Vitamin A (nmol/mg protein LDL)	Homosist. (μ mol/L)
1	36.6	3.0	39.6	5.9	0.37	19.6
2	33	3.5	36.6	7.0	0.39	13.7
3	39	2.15	28.4	3.6	0.6	18.5
4	27	3.3	33.2	4.0	0.4	22.1
5	34.8	2.7	30.6	4.2	0.3	10.6
6	46.7	2.15	33.4	5.9	0.4	22.5
7	33.3	4.7	50.0	6.1	0.39	13.9
8	43.2	2.1	38.4	6.2	0.31	14.4
9	30	2.1	37.1	10.2	0.41	17.0
10	70	2.5	31.6	4.8	0.37	16.1
11	66	2.6	24.6	6.1	0.38	15.9
12	50	1.87	23.7	7.5	0.56	12.0
13	30	2.7	37.0	6.5	0.56	11.2
14	22	3.0	31.4	4.8	0.55	14.5
15	25	4.3	42.0	5.0	0.18	13.4
16	33.3	6.0	45.0	4.8	0.34	17.3
17	41.2	3.4	39.1	6.5	0.35	13.3
18	38	4.0	33.3	6.0	0.75	10.8
19	35.1	2.9	38.9	4.0	0.4	10.8
20	34	3.2	36.7	4.8	0.4	13.9
Mean±SD	15±3.5	3.1±1.0	35±6.4	5.7±1.4	0.4±0.1	15±3.5

Table 2. Group 2 (Patient Sun/Brother) LDL oxidation parameters

Group no	Lag Phase (minute)	Maximum oxidation ratio (μmol/mg protein LDL minute)	Maximum CD concentration (nmol/mL)	Vitamin E (nmol/mg protein LDL)	Vitamin A (nmol/mg protein LDL)	Homosist. (μ mol/L)
1	36	2.3	31	5.7	0.44	7.1
2	40	3.0	44	4.4	0.38	10.0
3	72	3.6	30	10.8	0.41	15.0
4	56	1.4	28.2	5.5	0.41	14.3
5	55	2.4	42	6.5	0.40	12.0
6	37	3.3	40	3.9	0.35	10.0
7	34.5	1.9	23	3.6	0.39	6.2
8	50	1.6	27	4.8	0.32	10.6
9	108	2.2	30.2	5.2	0.35	8.1
10	83	2.5	34.2	5.9	0.72	14.3
11	34	1.8	21	6.5	0.35	11.9
12	30.9	3.6	43	4.8	0.50	9.0
13	64	2.4	42	6.1	0.38	12.0
14	80	2.4	35.2	3.7	0.58	8.7
15	100	2.3	31.6	5.7	0.30	7.9
16	51.6	3.5	42	6.2	0.25	11.1
17	58.1	2.7	35	4.3	0.37	9.5
18	61	2.6	37.1	5.2	0.29	8.8
19	48.7	3.0	34.6	4.5	0.38	9.0
20	57.3	2.6	36.7	5.4	0.45	11.9
Mean±SD	58±21	2.5±0.5	34±6.6	5.4±1.5	0.4±0.1	10±2.4

Lag Phase Rate

Comparing the lag phase between Group 1 and Group 2, it was found to be longer in Group 2 and the difference was significant (P=0.005). No significant difference was determined between Group 2 and the control group in terms of duration of lag phase (p=0.54).

Table 3. Group 3 (Control Group)) LDL oxidation parameters

Group no	Lag Phase (minute)	Maximum oxidation ratio (μmol/mg protein LDL minute)	Maximum CD concentration (nmol/mL)	Vitamin E (nmol/mg protein LDL)	Vitamin A (nmol/mg protein LDL)	Homosist. (μ mol/L)
1	100	1.7	25.7	10.2	0.7	12.0
2	64	1.4	25.6	5.4	0.5	10.6
3	86.6	2.2	30.2	5.9	0.4	16.0
4	85	2.7	35	7.7	0.8	16.4
5	28	2.7	36	6.1	0.14	8.3
6	83	2.2	31.6	3.5	0.5	6.4
7	76.6	2.2	33.2	5.4	0.4	8.1
8	534	2.3	28.4	6.2	1.2	9.0
9	55	2.6	41	5.9	0.38	7.8
10	50	1.3	30	6.0	0.7	14.0
11	76.6	1.5	25	5.3	0.9	7.3
12	50	1.6	25	4.8	0.4	10.3
13	48	1.2	25	5.8	0.14	10.0
14	67	1.5	24	6.7	0.6	8.1
15	83	1.2	19.5	7.0	0.6	7.6
16	66	2.4	24	6.0	0.4	9.7
17	67	3.0	39.1	5.2	0.38	8.9
18	75	2.7	33.3	5.1	0.4	10.1
Mean±SD	67±17	2±0.6	29.5±5.8	6.0±1.3	0.5±0.2	10±2.8

Table 4. intergroup evaluation of lag phase values (minute)

GROUPS	n	Mean	Range	SD
Group 1 (patient)	20	38.4	22.0-70.0	12.2
Group 2 (son/brother)	20	57.8	30.9-108.0	21.6
Group 3 (control)	18	67.4	28-100.0	17.7

Maximum oxidation rate

Comparing the maximum oxidation rate/minute between Group 1 and Group 2, it was found to be higher in Group 1, which was statistically significant (p=0.026). Comparing the sons and the control group in terms of maximum oxidation rate, it was found to be statistically significantly higher in Group 2 vs. Group 3 (p=0.017).

Table 5. intergroup evaluation of maximum oxidation values(μmol/mg protein minute)

GROUPS	n	Mean	Range	SD
Group 1 (patient)	20	3.1	1.87-6.0	1.0
Group 2 (son/ brother)	20	2.5	1.39-3.6	0.5
Group 3 (control)	18	2.0	1.2-3.0	0.5

Maximum CD concentration

No statistically significant difference was determined between maximum CD concentrations of Group1 and Group 2 (p=0.607). However, it was significantly higher in Group 2 as compared to Group 3 (control group) (p= 0.023).

Table 6. intergroup evaluation of maximum CD concantration values (nmol/mL)

GROUPS	n	Mean	Range	SD
Group 1 (patient)	20	35.5	23.7-50.0	6.4
Group 2 (son/ brother)	20	34.3	21.0-44.0	6.6
Group 3 (control)	18	29.5	19.5-41.0	5.8

Vitamin E and Vitamin A contents of LDL

No significant difference was determined between Group 1 and Group 2 (p=0.618) or between Group 2 and Group 3 (p=0.244) in terms of Vitamin E concentration.

Table 7. intergroup evaluation of vit E values (nmol/mg protein LDL)

GROUPS	n	Mean	Range	SD
Group 1 (patient)	20	5.7	3.6-10.2	1.5
Group 2 (son/ brother)	20	5.4	3.6-10.8	1.5
Group 3 (control)	18	6.0	3.5-10.2	1.3

Vitamin A content of LDL

No significant difference was determined between Group 1 and Group 2 (p=0.500) or between Group 2 and Group 3 (p=0.058) in terms of Vitamin A concentration.

Table 8. intergroup evaluation of vit A values (nmol/mg protein LDL)

GROUPS	n	Mean	Range	SD
Group 1 (patient)	20	0.42	0.18-0.80	0.13
Group 2 (son/brother)	20	0.40	0.25-0.7	0.10
Group 3 (control)	18	0.50	0.13-1.2	0.28

DISCUSSION and CONCLUSION

Family history of early-onset heart disease is defined by the American National Cholesterol Education Program (NCEP) as 'myocardial infarction or sudden cardiac death under the age of 55 years in the father or 1st degree male relatives or under the age of 65 years in the mother or 1st-degree female relatives'.

Given this definition, male patients with vascular lesion detected on coronary angiography in 9 Eylül University Department of Cardiology, as well as their 1st-degree male relatives, were evaluated in order to identify both the subjects with family history of cardiac disease and their 1st-degree relatives to form the study group.

Currently, there are numerous studies investigating LDL oxidation in the patient groups with coronary heart disease (CHD) (31.45.51). During LDL oxidation, extracellular composition of lipoprotein particles is unstable; therefore, continuous kinetic measurements or various analyses performed at different time intervals are necessary to explain the kinetics of oxidation process (1,3,5,10)..

Esterbauer et al. explained the characteristics of conjugated diene (CD) measurement, which as an *invitro* method is suitable also for *invivo* conditions as the indicator of susceptibility of LDL to

oxidation. CD is a method that gives critical information about preventive effects of kinetic changes and antioxidants (5).

In the present study, susceptibility of LDL to oxidation is measured after isolating the Apo B-containing lipoproteins using CuSo4 (6,17).

Duration of lag phase, maximum oxidation rate and maximum CD concentration, which are the indicators of susceptibility to oxidation, are evaluated in the study groups. Studies conducted with patient groups having coronary artery disease revealed different outcomes concerning these parameters. The reasons for this include both the heterogeneity of the study groups and non-standardized measurement methods. In the present study, duration of lag phase was shorter and maximum oxidation rate is longer in Group 1 (fathers) than Group 2 (sons/brothers). The groups were not different in terms of maximum CD concentration. Based on these results, it was determined that susceptibility of LDL to oxidation increased in this group with higher mean age and significant coronary disease.

Our target population was the 1st-degree male relatives under the risk of early-onset CHD, which is not investigated in the earlier studies. In addition, those with diabetes mellitus, hypertension, hyperlipidemia or chronic kidney disease, smokers, and those receiving antioxidant or lipid-lowering drugs were excluded from the study, and the gender was provided to be the same. This allowed the standardization of the groups by eliminating potential confounding factors. Karmansky et al. found no difference between the CHD group and the controls in terms of above-mentioned parameters. This was attributed to the group's being heterogeneous, to the medications' being not restricted, and to the above-mentioned methodological factors (17).

Comparing with the other fat-soluble vitamins, Vitamin E concentration in LDL is higher as 6/1. Together with ubikinon, Vitamin E and Vitamin A induce formation of lag phase and prevent LDL oxidation by removing the free radicals produced during this phase. However, it is completely consumed at the end of the lag phase and, therefore, LDL oxidation starts rapidly (12,22,57).

In the Cambridge Heart Antioxidant Study (CHAOS), Vitamin E (400-800 IU/dl) was given for a long time to more than 2000 patients with CHD diagnosed by coronary angiography, and statistically significant reduction was observed in the incidence of nonfatal myocardial infarction (2,16).

Dieber-Roteneder conducted a study by giving external Vitamin E preparation. In this study, however, they stated that vitamin E concentration in LDL is alone not adequate although it is a critical protecting factor and that its antioxidant effect shows variation among people. In a group consisted of 36 subjects, Vitamin E concentration in LDL and lag phase were measured and the correlation coefficient was found quite low as r=0.145, which was found to be r=0.51 following Vitamin E replacement. Although it was the effect of exogenous Vitamin E, 50% correlation was determined (16).

Factors that have an effect on the susceptibility of LDL to oxidation remain unclear. Although Vitamin E is considered to be the basic antioxidant in LDL, absence of correlation between lag phase and Vitamin E concentration in some studies suggests that there might be other factors, which include fatty acid concentration and composition in LDL, as well as structural and conformational variations. Specific Cu^{+}-binding areas on Apo B induce the formation of free radicals. It is thought that genetic alterations in Apo B may change the number and affinity of Cu^{+}-binding areas and the susceptibility of LDL to oxidation.

In the present study, there was no difference between the 1st-degree relatives of Group 1 (Group 2) and the control group (Group 3) in terms of Vitamin A and Vitamin E contents of LDL. Accordingly, duration of lag phase may be similar. Besides, Apo B LDL ratio and the size of LDL particles were similar between the two groups. Therefore, antioxidants in LDL protect the LDL against antioxidants among the 1st-degree relatives and the controls. However, oxidation after lag phase is faster among 1st-degree relatives vs. the control group and the amount of CD in this period is higher.

In conclusion, maximum oxidation rate and maximum CD concentration determined by measuring the susceptibility to kinetic oxidation via CD being significantly higher in the high-risk 1st-degree male relatives/sons having family history of early-onset CHD as compared to the control group indicates increased LDL oxidation in this group vs. healthy population. Such subjects are more likely to develop CHD as compared to the normal population. Taking preventive measures and monitoring the risky group beginning from the early ages may delay CHD and reduce patient expenditure.

Informed Consent: Patients were selected with the permission of Prof. Dr. Sema GÜNERİ, Head of the Department of Cardiology. Coronary Angiography reports were reviewed in the Department of Cardiology. If you have them using appropriate criteria and first-degree male relatives were called. Measurements were given on a voluntary basis and the results were reported to the patients.

Compliance with Ethical Standards: retrospective study

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - EO; Design - EO; Supervision - EO; Fundings - EO; Materials - EO; Data Collection and/or Processing - EO; Analysis and/or Interpretation - EO; Literature Search - EO; Writing Manuscript - EO; Critical Review - EO

Conflict of Interest: No conflict of interest was declared by the authors.

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