CORRELATION BETWEEN LAG TIME OF LDL TO *IN VITRO* OXIDATION AND *IN VIVO* OXIDIZED LDL IN THE PATIENTS WITH CORONARY ARTERY DISEASE

Hashem Nayeri[1], Gholam Ali Naderi[2], Ebrahim Javadi[3], Sedigheh Asgary[2], Abbas Lotfi[4], Masoumeh Sadeghi[2]

Abstract

BACKGROUND: The oxidative modification of low density lipoprotein (LDL) is believed to play an important role in the development of atherosclerosis. Thus, measurement of plasma oxidized LDL (OX-LDL) is essential for atherosclerotic diseases, for investigating its relevance to atherosclerotic diseases. We aimed to assess the oxidized LDL in patients with coronary artery disease and correlation between serum oxidized low density lipoprotein and in vitro susceptibility of LDL to oxidation.

METHODS: Subjects of the study were selected from patients who undergone angiography (42 patients with coronary artery disease and 40 controls without any evidence of CAD). The susceptibility of LDL to in vitro oxidation was assessed with the addition of a $Cuso_4$ solution. The lag time, propagation rate and maximal diene calculated from the oxidation curve. Biochemical factors (FBS, total cholesterol, TG, LDL, and HDL) were measured in these subjects. SPSS version 15.5 was used to analyze the data, P- value under 0.05 was considered to be significant.

RESULTS: The results indicated that the serum OX-LDL concentration was significantly elevated in CAD patients and the lag time was significantly shorter than controls ($P < 0.05$). These results clearly confirm that LDL from persons with CAD is more susceptible to oxidative modification in vitro than LDL from healthy subjects. The other measured biochemical factors were not significantly different between CAD patients and controls ($P > 0.05$). Correlation between serum OX-LDL and susceptibility of LDL to *in vitro* oxidation did not show significant association ($P > 0.05$).

CONCLUSION: our findings suggest that a high OX-LDL concentration and a short LDL oxidation lag time might be independent risk factors for CAD.

Keywords: OX-LDL; Lag time; Maximal Diene; Propagation Rate ;Susceptibility.

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Introduction

Atherosclerosis is a multifactor disease which is a primary cause of morbidity and mortality in the world. Recent theory suggests that atherosclerosis is a long-lasting inflammatory disease that oxidative modification of LDL is a key event in the oxidation hypothesis of atherogenesis.[1,2]

Oxidized LDL is directly involved in the initiation and progression of the atherosclerotic disease process, from the early-stage conversion of monocytes/macrophages into lipid-laden foam cells, to the late-stage development of coronary artery stenosis, plaque instability, plaque rupture, coronary thrombsis, and myocardial infarction.[2,3]

Circulating oxidized LDL is made by oxidation in the arterial wall by cell-associated lipoxygenase and/or myeloperoxidase.[4]

A causal role of modified LDL in the coronary artery disease (CAD) is supported by data from *in vitro*, animal, and human clinical studies.[5–8]

Oxidized LDL induces atherosclerosis by stimulation of monocyte infiltration and smooth muscle

1) PhD Student of Biochemistry, Science and Research Branch, Islamic Azad University, Tehran, Iran.
E-mail: hnaieri@gmail.com
2) Isfahan Cardiovascular Research Center, Applied Physiology Research Center, Isfahan University of Medical Sciences, Isfahan, Iran.
3) Department of Biochemistry, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran.
4) Department of Biochemistry, School of Medical Sciences, Tarbiat Modarres University, Tehran, Iran.

Corresponding author: *Hashem Nayeri*

cell migration and proliferation. It contributes to atherothrombosis by inducing endothelial cell apoptosis, and thus plaque erosion, by impairing the anticoagulant balance in endothelium, stimulating tissue factor production by smooth muscle cells, and inducing apoptosis in macrophages.[2]

Therefore LDL oxidation is considered to have a major importance in the pathogenesis of atherosclerosis.[9,10]

Oxidation of LDL is a lipid peroxidation process driven by free radicals which converts polyunsaturated fatty acids to lipid peroxide.[4,11] The peroxidation of LDL exhibits three following phases; initiation (conjugated dienes formation), propagation (peroxides formation), and decomposition (aldehydes formation) phases.[12]

Some studies suggest that in vitro induced lipoprotein oxidation (lipoprotein oxidizability induced by cu^{+2} and copper ions or by AAPH), mimics the in vivo lipid peroxidation conditions in this system.[13]

This study performed to compare the oxidized LDL in patients with coronary artery disease with healthy controls and correlation between serum oxidized low density lipoprotein (OX-LDL) and in vitro susceptibility of LDL to oxidation.

Materials and Methods

Subjects were selected from male patients who had undergone diagnostic coronary angiography using simple sampling method.

They were divided into two group of coronary artery disease (CAD), defined as at least 70% stenosis in one or more major epicardial arteries, and patients without any evidence of stenosis in their major coronary arteries (healthy controls). The exclusion criteria were as follows; triglyceride concentration above 2.3 mmol/l, diabetes mellitus, malignant disease, thyroid disease, smoking, dyslipidemia, age younger than 40 years or older than 60 years, familial hypercholesterolemia, alcohol abuse, renal disease, myocardial infarction or coronary artery bypass grafting in the 6 weeks prior to angiography and lipid lowering medication. Information on life style, medication and family history was obtained by direct interview.

The final sample size was 42 patients with and 40 without evidence of CAD. All subjects Included in the study signed informed consent forms. The study was approved by the Ethics Committee of Isfahan Cardiovascular Research Center.

Fasting blood samples (18 ml) were obtained by venipuncture before Angiography. Serum samples were separated by low speed centrifugation (3000 x g for 15 minutes) and used for measurement of lipids profiles, OX-LDL and LDL oxidizability. All measurements were carried out on fresh serum, except for LDL isolation and LDL susceptibility to oxidation

Plasma cholesterol, triglyceride, high density cholesterol (HDL-C), low density cholesterol (LDL-C), and fasting blood sugar levels were determined using an automated enzymatic assay by auto-analyzer Hitachi 902 and using special kits (Diasys Diagnosis Inc., Holzheim, Germany) performed by Pars Azmon (Pars Azmoon, Tehran, Iran).

A competitive ELISA kit (Mercodia, Uppsala, Sweden) was used to determine OX-LDL concentrations in serum. Intra-assay and inter-assay CVs were 6.7% and 7.0%, respectively. Results are expressed in arbitrary units as U/L.

Isolation of LDL

The plasma solution was then distributed into 10 ml polycarbonate centrifuge tubes and a discontinuous density gradient was made by overlaying the plasma solution (2.8 ml) with 6.6 ml A Solution containing (11.4 g NaCl, 0.1g Na_2- EDTA, 1cc NaOH total volume =1003 cc, d = 1.006 g/ml).

The tubes were ultracentrifuged in Beckman Coulter optima L-100XP ultracentrifuge equipped with a 90 Ti fixed angle rotor, at 60000 rpm for 6 h at 16°C. After centrifugation, the tubes were carefully removed from the rotor and placed in the vertical position. The VLDL fraction appears as a white band at the supernatant. After isolation VLDL and solution in upper layer tube, residual content of tube mixed with B solution contained 24.8 g NaBr + 100 cc A solution.

The tubes were ultra centrifuged at 60000 rpm for 12h at 16°C. After centrifugation, The LDL fraction appears as a yellow-orange band at the supernatant. The LDL fraction was collected and dialyzed in a glass suction apparatus filled with phosphate buffer (21.1mM Na2 HPo4 + 17.7 Na H2Po4 + 160 mM NaCl pH = 7.4) in the dark at 4°C.[14] Protein concentration was determined by the method of Lowy et al.[15]

LDL oxidation

LDL (150 µg protein/L) was incubated with 5 µmol CuSO4/L for 5h at room temperature. The formation of conjugated dienes was monitored continuously by measuring the increase in absorbance at 234 nm.[2] Incubations were carried out in the spectrophotometer cuvette. LDL oxidation was recorded every 10 min.

The lag time required for the initiation of lipoprotein oxidation was calculated from the oxidation curve.

Statistical analyses

All quantitative data are presented as the mean ± SD. Statistical analysis was performed using SPSS version 15.5. The student t-test, Pearson´s rank correlation coefficient and binary logistic regression tests were used for the processing of obtained data.

Results

No significant differences were found in the levels of FBS, total cholesterol, TG, LDL, and HDL cholesterol in the patients. Whereas the OX-LDL concentration were significantly elevated in comparison with the controls ($P < 0.05$) (Table 1), Protein concentration of LDL were not significantly different in patient and control subjects.

Susceptibility of LDL to in vitro oxidation is presented in Table 2. A significant difference was found in lag time between CAD patients and controls. The maximal diene and propagation rate did not show any statistical difference between two groups.

To reveal the possible association between susceptibility of LDL oxidation and level of *in vivo* OX-LDL, we analyzed the relation between *in vivo* OX- LDL level in CAD patients and characteristics of *in vitro* LDL oxidation in these subjects. Neither the amount of maximal diene nor the propagation rate of LDL oxidation was found to be related with plasma OX-LDL.

We also found a significant negative relation between the OX-LDL levels and lag time of LDL.

Table 1. Plasma concentrations of lipids and apoproteins (mean ± SD) in CAD and control subjects

Parameter	CAD (n = 42)	Controls (n = 40)	P-Value
Triglycerides (mg/dl)	158.1 ± 53.4	162.7 ± 59.6	0.71
Total cholesterol (mg/dl)	183.3 ± 30.7	175.3 ± 34.3	0.28
LDL cholesterol (mg/dl)	107.6 ± 21.1	104.9 ± 24.6	0.61
HDL cholesterol (mg/dl)	38.5 ± 8	36.3 ± 8.8	0.52
FBS(mg/dl)	93.4 ± 22.5	93.9 ± 17.1	0.9
OX-LDL(U/L)	171.6 ± 102.9	131./2 ± 60.1	0.033*

* CAD and control subjects were significantly different in each analyzed parameter ($P < 0.05$).

Table 2. Characteristics of absorbance curves during oxidation of total LDL from the CAD and control subjects

Parameter	CAD (n = 42)	Controls (n = 40)	P-Value
Lag time (min)	101.06 ± 15.3	114.7 ± 30.9	0.03*
Maximal diene (µmol/mg LDL protein)	3.3 ± 1. 5	3.1 ± 1.6	0.55
Propagation rate (nmol /mg LDL protein/min)	221.8 ± 100.6	203.8 ± 91.18	0.46

*CAD and control subjects were significantly different in each analyzed parameter ($P < 0.05$).

Discussion

This study shows total cholesterol, TG, LDL and HDL cholesterol aren't significantly different between control and CAD patients. These results indicate that however, serum levels of LDL-cholesterol are an important risk factor in the development of atherosclerosis but is a poor predictor of a future coronary heart disease (CHD).

The OX-LDL concentrations in patients are significantly elevated in comparison with the controls. Therefore OX-LDL was shown to be the strongest predictor of future CHD compared to conventional lipoprotein profiles and other traditional risk factors.

Meisinger et al (2005) showed that elevated blood levels of OX-LDL were the strongest predictor of future CHD events compared to a conventional lipoprotein profile and other traditional risk factors for CHD.[16] When blood levels of OX-LDL and LDL-cholesterol were compared for their ability to identify patients with CAD, in the study by Johnston, a strong positive correlation between blood levels of OX-LDL and patients with CAD was found, far beyond that of LDL-Cholesterol.[3]

A significant decrease in lag time of CAD patients shows that LDL from persons with CAD is more susceptible to oxidative modification *in vitro* than LDL from healthy subjects. This result is supported by other studies.[17–20]

This study did not show significant association between *in vivo* OX-LDL and susceptibility of LDL oxidization in the patients with CAD. In a study by Jun et al, there was a significant association between *in vivo* OX-LDL and susceptibility of LDL oxidization in the patients with CAD.[21] Comparison between this study and Jun et al study demonstrates that LDL in the patients with CAD is more susceptible to oxidative modification.

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