KINETIC ANALYSIS OF LDL OXIDATION IN IHD AND IHD RISK SUBJECTS IN INDIAN POPULATION

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ABSTRACT

High plasma concentration of low-density lipoprotein (LDL) is associated with increased risk of atherosclerosis. Modified forms of LDL, especially oxidized LDL play a major role in its pathogenesis. This article gives detailed insight into the kinetics of *in vitro* LDL oxidation by copper at different concentrations in normal and high-risk group subjects. Basal level of oxidatively modified LDL was significantly higher in ischaemic heart disease (IHD) and IHD hyperlipidemic subjects compared to normolipidemic and hyperlipidemic control subjects, respectively. Derivatization of amino groups of apo-lipoprotein as monitored by estimating free amino groups concentration, was significantly higher in high-risk group and established IHD cases. Kinetics of oxidation was studied with two different concentrations of CuSO_4 (2.5 mM and 7.5 mM). Thiobarbituric acid reactive substances (TBARS) level increased with time, and up to 95% oxidation was observed in 8 hr. About 60-65% less free amino groups were observed in native-LDL isolated from IHD patients compared to normal subjects. Study also showed an increase in two oxidative products studied, 20α -OH-cholesterol and 4-cholesten-3-one with oxidation time accompanied by corresponding decrease in LDL cholesterol. Increase in oxidative species was more evident in high-risk group and IHD patient. Basal level of oxidatively modified LDL measured in terms of TBARS was significantly higher in present study, strongly support that the extent of LDL oxidation monitored as TBARS and FAG level in circulating-LDL could be used as risk marker for high risk group.

KEYWORDS

LDL, ox-LDL, Kinetics, FAG, Cholesterol, HPLC

INTRODUCTION

Ischaemic heart disease could be diagnosed as chest pain arising from the heart, usually under the sternum (breastbone), due to an inadequate supply of oxygen to the heart muscle. The commonest cause is coronary-artery disease with partial blockage caused by a cholesterol plaque or atherosclerosis. Atherosclerosis is conceptually defined as the result of a multiplicity of interactive cascades among injurious stimuli and the healing responses of the arterial wall, occurring concurrently within a hyperlipidemic environment. It is a complicated process that involves the arterial wall cells, including monocyte-derived macrophages, platelets and plasma lipoproteins. Recent clinical and experimental studies of various kinds have

established that elevated plasma concentrations of low-density lipoprotein (LDL) are associated with accelerated atherogenesis (1-3). Macrophages are especially adopted for cholesterol accumulation derived from cell debris, from platelets, and mainly from plasma lipoproteins, particularly LDL (4). Various LDL modification including chemical, enzymatic, and arterial cell mediated, were shown to result in modified forms of LDL associated with lipid oxidation and amino acid derivatization, which led to recognition by the Acetylated-LDL receptor and to macrophage cholesterol accumulation.

One of the processes involved in atherogenesis that has been studied significantly both *in vitro* and *in vivo* is the oxidation of LDL. A number of oxidation products of lipids and proteins have been demonstrated in atheroma (5-9). Small quantities of lipoproteins having many of the characteristics of oxidized LDL circulate in human plasma. The potential proatherogenic effects of oxidized LDL are numerous: oxidized LDL can attract monocytes,

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induce monocyte adhesion to endothelium, promote foam cell formation, injure cells, induce migration and proliferation of smooth muscle cells, interfere with endothelium-mediated relaxation, and promote procoagulant properties of vascular cells (10). Much efforts has been devoted to gaining understanding of the kinetics of LDL oxidation induced by free radical generators, enzymatic reaction and transition metals. Large body of evidence supports the view that some oxidative modification of LDL contributes to atherogenesis in a variety of animal models. Attempts are being made to determine whether oxidation markers can be linked to active arterial disease (7, 8).

There are a few studies on kinetics of LDL oxidation in Western population, but no comparative study reported in Indian population. Moreover, studies are not comprehensive enough to give insight into risk groups. As coronary heart disease are on the rise in Indian population the present investigation was aimed at studying kinetics of LDL oxidation in the Indian population with high risk of Ishaemic heart disease (IHD) in an attempt to use this tool as an epidemiological marker for high risk group.

MATERIALS AND METHODS

Selection of Subjects

The study was conducted in four groups of human subjects as outlined below: Group I: Healthy control subjects with no history of any coronary disease. Group II: Hyperlipidemic control subjects (Atherogenic index more than 5.0, triglycerides (TG) > 160 mg/dl) but with no prior history of coronary disease. Group III: Established IHD cases with normal lipid status. Group IV: Established IHD cases with hyperlipidemic status. Age and sex matched control subjects were selected either from the relatives of the patients or from hospital staff with their consent. These cases were thoroughly screened with regards to IHD, diabetes mellitus, and hypertension or for any other significant illness. IHD and IHD hyperlipidemic subjects were selected from indoor and outdoor patients attending G.B. Pant Hospital. In these two groups patients were also screened for any associated diseases like diabetes mellitus, liver disorders and other chronic diseases or any drug medication affecting the study and such patients were excluded.

Collection of Blood Sample and Isolation of Plasma

All subjects were instructed to observe an overnight fast for 12 hr prior to the venipuncture. The sample was collected after making the subject sit in sitting position quietly for 5 min to avoid rapid transient decrease in triglycerides due to stress. The sample was transferred to a glass tube after removing the needle from the syringe to prevent hemolysis. About 10 ml of blood was collected aseptically in heparinized syringe for LDL studies. Blood samples were centrifuged (Remi R-8 Lab Centrifuge) at 3000 rpm (1000g) for 5 minutes to separate plasma as supernatant for further analysis.

Measurement of Lipid Profile

Total plasma cholesterol, triglycerides and high-density lipoprotein (HDL)-cholesterol were analyzed using Olympus Auto-Analyzer analytical system and represented as mg/dl. Total HDL-cholesterol was isolated from plasma according to Burnstein *et al.* (11). LDL-cholesterol was calculated according to Friedwald's formula [LDL-cholesterol = Total Cholesterol - HDL-Cholesterol - TG/5].

Isolation of LDL

LDL was isolated from plasma by density gradient ultracentrifugation (12); all the solutions contain 1 mM EDTA to prevent oxidation. The ultracentrifugation was carried out at 250,000g (SW-28 rotor, Beckman L7-80 ultracentrifuge) for 2 hr at 10°C. Fractions were collected from the top of the tube by a syringe. Total content of tubes were fractionated into eight fractions. Each fraction was analyzed for total cholesterol, proteins, triglycerides and HDL/LDL-cholesterol. The amount of protein content in LDL samples was estimated using Bradford's reagent. Purity of LDL containing fractions was assessed by SDS-polyacrylamide gel electrophoresis. Pooled LDL fractions were dialysed overnight at 4°C in PBS against two changes. LDL content was concentrated by dialysing against PEG 8000 and used for further analysis.

Oxidation of LDL

In vitro oxidation of LDL was carried out by incubating it in the presence of 2.5 μ M or 7.5 μ M CuSO₄ at 37°C for 0-24 hr. The oxidation was stopped by the addition of 1mM EDTA and freezing the samples.

Estimation of Malondialdehyde

Malondialdehyde (MDA) has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid (TBA) to give a red species absorbing at 535 nm (13).

Estimation of Free Amino Group

Free amino groups (FAG) in LDL were measured using trinitrobenzenesulfonic acid (TNBS) method (14). For the estimation of FAG we used Lysine as standard, so the value of FAG are represented as nmol Lys/mg LDL protein.

Estimation of Oxidized Species

HPLC was used to identify and quantify the oxidized cholesterol species in LDL with minor modifications in the method of Liu *et al.* (15). LDL samples were suspended in 2:1 (v/v) chloroform: methanol, and extracted overnight at 4°C. The lipid residue was re-suspended in hexane: isopropanol (95:5, v/v). The re-suspended samples were analyzed by HPLC (Waters, USA) employing a Water μ Porasil column (3.9 mm x 300 mm) at 1.5 ml/min flow rate in hexane: isopropanol (95:5, v/v) mobile phase. Cholesterol and its oxidation products were detected at 208 nm by Waters 484 tunable absorbance detector.

RESULTS AND DISCUSSION

Serum lipid profile was determined after an overnight fast in patients and control subjects as mentioned in Materials and Methods. There was no significant difference between the male and female subjects of the groups (data not shown), so in all the groups both male and female subjects were grouped together. For comparison, control subjects were selected from those having cholesterol levels less than 250 mg/dl and TG level less than 160 mg/dl and AI<5, with no recorded case history of IHD. In high risk group hyperlipidemic subjects, IHD and IHD hyperlipidemic subjects mean cholesterol level was found to be elevated about 2-3 folds compared to normal control subjects.

LDL fractions from all the four different groups were isolated by ultracentrifugation. Each fraction was subjected to analysis of lipid profile by auto-analyzer and was also subjected for 4-12% gradient SDS-PAGE. Fractions, which contained maximum LDL-cholesterol, were found to have mainly a single band as detected by Coomassie brilliant blue staining of the gel (data not shown). These fractions were pooled and used for oxidation studies.

Oxidation of LDL

Few groups have made the systematic comparison of oxidation levels in different lipoproteins of human

plasma. For measuring the oxidation level in LDL we have used two methods, employing a colorimetric agent, TBA to measure the MDA level and in others we have used TNBS to detect the derivatization of free amino groups of lipoproteins during oxidation. MDA which is produced during lipid peroxidation has been found to be reactive to TBA and gives the color which can be measured by spectrophotometer. TBARS have been reported in VLDL, LDL, and HDL, but the LDL fraction always contained the highest proportion.

We have undertaken this study, as there was no comparative data available on Indian population. We isolated LDL from all the four groups described earlier and subjected LDL (n=50) for oxidation with CuSO_4 for different duration at 37°C. It was observed that the level of TBARS in native LDL in the control group was 2.52 ± 0.82 nmol MDA/mg LDL-protein that increased to 32.03 ± 3.13 nmol MDA/mg LDL-protein after incubation with 7.5 mM CuSO_4 for 24 hr (Fig. 1). In hyperlipidemic control group native LDL was 3.72 ± 0.81 nmol MDA/mg LDL-protein that increased to 33.29 ± 5.29 nmol MDA/mg LDL-protein after incubation with 7.5 mM CuSO_4 for 24 hr. TBARS level in LDL derived from IHD and IHD hyperlipidemic subjects showed 2.37 fold and 1.17 fold increase, respectively (Fig.1) compared to their respective controls ($p < 0.001$). However, when compared with normolipidemic control group, the increase was 2.54 fold for the IHD hyperlipidemic group ($p < 0.001$).

We have observed thirteen fold increase in TBARS level after oxidations with 7.5 μM CuSO_4 in normolipidemic control subjects. Though fold increase was less than earlier reported (17, 18). This may be due to difference in basal level of TBARS, as we observed a higher level in native LDL. We observed 8.90 fold increase in TBARS level in hyperlipidemic group, 5.84 fold in IHD group and 5.54 fold in IHD hyperlipidemic group after exposure of native LDL to 7.5 mM CuSO_4 . This difference may also be due to higher concentration of CuSO_4 (10 mM) used by Frostegard *et al* (17), another possibility could be difference in the eating behaviour of Indian subjects, which might have resulted in higher level of TBARS in native LDL. There is little data on lipid peroxide level and oxidative predisposition of plasma LDL from coronary disease patients. Chiu *et al.* (16) could not detect any significant difference between the TBARS level of LDL of normolipidemic control and CAD patients.

Kinetics of LDL Cu²⁺-Mediated Oxidation

The time course of *in vitro* LDL oxidation is a rather complex summation of different reactions that can be roughly divided into three phases, a lag, a propagation and a decomposition phase. During the lag phase the endogenous LDL antioxidants are consumed and no significant oxidation of unsaturated fatty acids (PUFAs) take place. When LDL is depleted of its antioxidants, PUFAs are rapidly oxidized to lipid hydroperoxides (propagation phase), which are then converted to a variety of other products including reactive aldehydes (decomposition phase). Enhanced *in vitro* susceptibility for oxidation of lipoproteins derived from hypercholesterolemic patients is thus, probably, the result of both a reduced content of antioxidants and increased concentration of lipoprotein cholesterol and arachidonic acid.

We studied the kinetics of LDL oxidation in four subject groups (normolipidemic control, hyperlipidemic control, IHD and IHD hyperlipidemic patients). It is evident from the data that the TBARS value increased with time and reached a plateau after a period of 24 hr of oxidation with a little increase thereafter (Fig. 2A). Moreover, more than 90% of the oxidation is achieved in all the groups by 8 hr of incubation with 7.5 μ M CuSO₄. A strong variation in the lag phase, and therefore, in the oxidation

resistance of LDL has been demonstrated in healthy subjects and in patients.

It was observed that the percent native oxidation (percent of maximum MDA value obtained in the group) in normolipidemic control, hyperlipidemic, IHD and IHD hyperlipidemic groups was 7.86, 11.23, 17.11 and 18.02 respectively (Table 1). After 6 hr of oxidation with 7.5 μ M CuSO₄ there was not much difference in normolipidemic control and IHD group (90.91 and 93.84, respectively) and neither in hyperlipidemic and IHD hyperlipidemic group (87.59 and 87.90, respectively). If we go by absolute value of MDA, we did observe a difference in the level of oxidation product with time to varying extent in different groups (Fig 2A). Steinbrecher and co-workers (18) reported a time dependent increase in the LDL TBARS incubated with 5 μ M CuSO₄ up to 20 hr. Similarly, Lavy *et al.* (19) in a time course study of oxidation of LDL showed a substantial increment in oxidation between 6 and 24 hr of incubation with 10 μ M CuSO₄. Further, they observed the enhanced oxidation of hypercholesterolemic LDL in comparison to control LDL after 24 hr, but the difference was not apparent after 6 hr of incubation, though their results also showed a increase in the TBARS values in the hypercholesterolemic LDL compared to control LDL throughout the time course of LDL oxidation.

Table 1 : Relative kinetics (Susceptibility) of LDL oxidation following incubation with 7.5 μ M CuSO₄ (MDA % maximal value) in different groups.

Time (hr)	Normal n=4	Hyperlipidemic n=4	IHD n=4	IHD Hyperlipidemic n=4
Native	7.86	11.23	17.11	18.02
0	7.99	11.35	17.20	18.17
0.25	10.45	14.26	17.40	18.73
0.50	21.35	19.40	20.97	20.95
1	30.81	25.14	30.28	33.61
2	39.27	37.84	43.27	40.75
3	46.17	43.04	50.68	49.45
4	69.34	58.15	73.09	68.88
5	79.48	66.83	83.17	78.08
6	90.91	87.59	93.84	87.90
8	96.37	96.63	97.42	96.79
24	100	100	100	100

To compare the effect of concentration of Cu^{2+} on the initiation and progress of LDL oxidation we compared level of oxidation with lower concentration of Cu^{2+} (2.5 mM CuSO_4). We observed a tremendous difference in the level of TBARS after 24 hr oxidation of LDL. There was a longer lag phase in control group as compared to IHD patients and in hyperlipidemic control subjects as compared to IHD hyperlipidemic group with 2.5 μM CuSO_4 concentration from 0 to 24 hr study time (Fig. 2B). We did not observe such a difference when we used 7.5 μM concentration of CuSO_4 from 0 to 24 hr in all the four groups. Cominacini *et al.* (20) did a LDL kinetic study in CAD, type IIa hyperlipoproteinemia and controls by incubation with 50 mM CuSO_4 for 5 hr. They found the value of the lag phase significantly higher in controls than in type IIa hyperlipoproteinemia patients which was itself higher than in CAD patients. Chiu and co-workers (16) also did a kinetic study of LDL oxidation in control and CAD group by varying the concentration of CuSO_4 used for oxidation, and studied the oxidation up to 5 hr at varying time intervals. They reported a significantly greater TBARS levels in CAD patients than in control after incubation for 5 hr with 2.5 and 5 μM CuSO_4 .

There was a significant difference in the percent native oxidation value after 6 hr of oxidation between normolipidemic control and IHD group (62.1% and 80.0%) and hyperlipidemic control and IHD hyperlipidemic group (74.4% and 87.2%). The difference after 8 hr of oxidation was little less between normolipidemic control and IHD group (86.2 and 91.4) and hyperlipidemic and IHD hyperlipidemic group (87.2 and 94.6), respectively. Our results indicate that increase is dependent on concentration of copper used for oxidation, similar observations were made by Steinbrecher *et al.* (18). We obtained 91% oxidation in same time with 7.5 μM CuSO_4 and 62% oxidation with 2.5 mM CuSO_4 . But, on the other hand, Lavy *et al.* (19) did not observe significant increase up to 6 hr of LDL incubation with 10 μM CuSO_4 . They observed the increase only after 6 hr of incubation. The difference could be due to higher amount of LDL used by these workers for oxidation.

We have observed longer lag phase in normolipidemic control group compared to hyperlipidemic, IHD and IHD hyperlipidemic group, when LDL was incubated with 2.5 μM CuSO_4 concentration. This may be due to more antioxidant

in control subjects compared to IHD and risk subject group. Cominacini *et al.* (20) have reported increased lag phase when LDL was incubated with 50 mM CuSO_4 but, Chiu *et al.* (16) have reported longer lag phase in control compared to the CAD patient group when LDL was oxidized with 2.5 μM CuSO_4 but they did not observe such delay when 10 μM of CuSO_4 was used for LDL oxidation. Our data support these observations made earlier.

Measurement of Free Amino Groups

In vitro oxidation of LDL results in change in a number of properties like, increase in content of oxidized forms of cholesterol, increase in negative charge, decreased content of polyunsaturated fatty acids and derivatization of lysine residues.

In order to study the derivatization of free amino groups during the course of oxidation of LDL we measured FAG using TNBS. We observed a significant decrease in the TNBS reactivity of oxidized-LDL as compared to native LDL after exposure to 7.5 μM CuSO_4 for 24 hr at 37°C, in all the four that we studied. In the normolipidemic control, LDL oxidized by 7.5 μM CuSO_4 at 37°C for 24 hr the FAG in native LDL decreased from 1990.20 ± 50.39 nmol Lys/mg LDL protein to 895.70 ± 34.49 nmol Lys/mg LDL protein after oxidation. TNBS reactivity of the hyperlipidemic group (731.50 ± 90.12 nmol Lys/mg LDL protein) was compared to the control group (895.70 ± 34.49 nmol Lys/mg LDL protein) after LDL oxidation with 7.5 μM CuSO_4 for 24 hr at 37°C (Fig. 3). Cominacini *et al.* (20) studied the free amino group level in LDL oxidized in the presence of 50 μM CuSO_4 and reported that during the oxidation of LDL there was a progressive decrease in the free amino groups (TNBS reactivity of LDL). Lavy *et al.* (19) also found a reduction in the TNBS reactivity in hypercholesterolemic as compared to normolipidemic controls after LDL was oxidized in presence of 10 mM CuSO_4 for 24 hr at 37°C. Liu and co-workers (15) did not find significant difference in the free amino group content in the native state between the control and coronary disease patient group. But, they have reported significant change in both the groups as a result of 50 μM CuSO_4 treatment for 60 min at 37°C, these findings are in accordance with our results, as with 7.5 μM CuSO_4 treatment of LDL for 60 min at 37°C we got a significant change in the normolipidemic control group (from 1991.24 ± 50.39 to 1757.50 ± 48.04 nmol Lys/mg LDL protein) as well as for the IHD group

(from 1481.25 ± 13 to 1365.00 ± 12.90 n mol Lys/mg LDL protein). Chiu *et al.* (16) also reported a significant decrease in the TNBS reactivity in the coronary artery disease patient as compared to the control after incubation of LDL for 5 hr with $5 \mu\text{M}$ CuSO_4 . Our results show a similar pattern when we compared to the TNBS reactivity of the IHD group (502.80 ± 23.27 n mol Lys/mg LDL protein) to the control group (895.70 ± 34.49 n mol Lys/mg LDL protein) after LDL oxidation with $7.5 \mu\text{M}$ CuSO_4 for 24 hr at 37°C .

Kinetics of Reduction of FAG

Kinetics of LDL oxidation studied in terms of reduction of FAG in all the four groups showed that the TNBS reactivity decreased up to 24 hr with no decrease thereafter which is in corroboration with TBARS measurements, moreover the decrease in the TNBS reactivity was maximum up to 8 hr with very less decrease observed after that up to 24 hr with $7.5 \mu\text{M}$ CuSO_4 concentration (Fig. 4). The FAG values observed after 24 hr oxidation of LDL were significantly lower at $7.5 \mu\text{M}$ Cu^{2+} than at 2.5 mM Cu^{2+} concentration in all the groups.

There was less decrease in the TNBS reactivity with longer lag phase in the normolipidemic and hyperlipidemic control group as compared to the IHD and IHD hyperlipidemic group with $2.5 \mu\text{M}$ CuSO_4 concentration from 0 to 24 hr study time. This may be due to higher level of antioxidants present in normolipidemic subjects. We did not observe the apparent difference in lag phase when we used $7.5 \mu\text{M}$ CuSO_4 for oxidation in all the four groups. Steinbrecher and co-workers (18) studied the decrease in kinetics of free amino groups during LDL oxidation and found a time dependent decrease in the TNBS reactivity on incubation of LDL with 5 mM CuSO_4 , which is comparable with the values we observed on using $7.5 \mu\text{M}$ CuSO_4 for LDL oxidation. Cominacini *et al.* (20) have reported decrease of 50% TNBS reactivity after 5 hr of LDL oxidation in presence of $50 \mu\text{M}$ CuSO_4 . We observed about 60% TNBS reactivity after 5 hr of LDL oxidation in presence of $7.5 \mu\text{M}$ CuSO_4 concentration. They have further reported very less decrease up to 90 minutes of oxidation of LDL in presence of $50 \mu\text{M}$ CuSO_4 , we observed that when we used lesser concentration of CuSO_4 ($2.5 \mu\text{M}$) for oxidation of LDL there was slow decrease in FAG. Chiu and co-workers (16) reported a significant decrease in the TNBS reactivity in the coronary artery disease patient as compared to the control

when 2.5 and $5 \mu\text{M}$ CuSO_4 concentration was used for oxidation of LDL, but did not observe the difference when 1 or $10 \mu\text{M}$ CuSO_4 was used for LDL oxidation. Our observations support these results, as we also got significantly greater decrease in IHD group as compared to the control group when we used $2.5 \mu\text{M}$ CuSO_4 concentration for LDL oxidation but not when we used $7.5 \mu\text{M}$ CuSO_4 concentration for LDL oxidation. In IHD hyperlipidemic and hyperlipidemic group there was greater TNBS reactivity in the former group when we used $2.5 \mu\text{M}$ CuSO_4 than $7.5 \mu\text{M}$ CuSO_4 for LDL oxidation.

Cholesterol Oxidative Species

In vitro oxidation of LDL results in an increased content of oxidized forms of cholesterol, derivatization of lysine amino groups, decreased content of polyunsaturated fatty acids and an increased negative charge and density. The oxidative species of cholesterol identified in our study by HPLC were 4-cholesten-3-one and 20- α -OH-cholesterol. The content of 4-cholesten-3-one and 20- α -OH-cholesterol were significantly higher in the patient groups as compared to their respective control groups ($p < 0.001$). Mean 20- α -OH-cholesterol level in normolipidemic control subjects was $11.5 \pm 6.28 \mu\text{g/mg}$ LDL protein, whereas in IHD patient mean level was $43.46 \pm 19.63 \mu\text{g/mg}$ LDL protein. In hyperlipidemic control subjects level was 21.28 ± 9.22 and in IHD hyperlipidemic it was $56.06 \pm 20.51 \mu\text{g/mg}$ LDL protein. Mean 4-cholesten-3-one level in normal was $1.61 \pm 0.84 \mu\text{g}$ and hyperlipidemic content it was $3.95 \pm 1.94 \mu\text{g}$, whereas in IHD and IHD hyperlipidemic patient level was $5.35 \pm 3.03 \mu\text{g}$ and $8.06 \pm 4.73 \mu\text{g/mg}$ LDL protein, respectively.

The non-oxidized LDL-cholesterol in the study groups was also found elevated in the HPLC measurements confirming the enzymatic analysis. The kinetics of LDL oxidation showed a time dependent increase in the oxidative species (4-cholesten-3-one and 20- α -OH-cholesterol) and a decrease in the non-oxidized cholesterol percent. We studied the kinetics of generation of cholesterol oxidation products using HPLC in the control subjects only. Here data is presented as the percent, all the species were added and taken as 100%, from this the species calculated. Cholesterol decreased from 93.41% to 65.62% after 24 hr of oxidation. 20- α -OH-cholesterol increased from 5.93% to 29.05% and 4-cholesten-3-one from 0.65% to 5.32% during the same period (Fig. 5). Liu *et al.*

(15) in their study have reported change in the oxidative profile of the LDL in coronary disease patients in the cholesterol component. The content of 4-cholesten-3-one in LDL from coronary disease patients was significantly elevated over that of control subjects. Non-oxidized cholesterol was also elevated in coronary disease patients. Other studies have identified 7- α -OH-cholesterol, 7-keto-cholesterol (21) and 26-OH-cholesterol (22) in plasma from healthy individuals. After incubation of cardiac sarcolemma vessels with cholesterol oxidase, 4-cholesten-3-one was the predominant species of oxidized cholesterol produced (23).

In conclusion, the present data strongly indicate that there is increased LDL cholesterol associated with IHD and high risk group, in addition to this *in vivo* and *in vitro* oxidation level showed increased susceptibility to copper induced oxidation in high risk group. With careful estimation these markers could be used as risk assessment tool in Indian population as the increase in oxidized LDL in hyperlipidemic group was statistically significant.

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Figure 1. : Shows the level of TBARS in the native and oxidized LDL (after 24 hr of oxidation with 7.5 mM CuSO_4) in normal, hyperlipidemic, IHD and IHD hyperlipidemic subject groups (n=50).

Figure 2. Kinetics (susceptibility) of oxidation of LDL. LDL was isolated from normal, hyperlipidemic, IHD and IHD hyperlipidemic subjects (n=4). LDL was oxidized with for up to 24hr in the presence of (A). 7.5 mM CuSO₄ (B). 2.5 mM CuSO₄ and level of TBARS measured as MDA generated. The lag phase in control group is more evident in figure B with low concentration of copper.

Figure 3. Shows the level of FAG, in the native and oxidized LDL in different groups (n= 50) after 24 hr of oxidation with 7.5 mM CuSO₄.

Figure 4. Kinetics (susceptibility) of oxidation of LDL isolated from normal, hyperlipidemic, IHD and IHD hyperlipidemic subjects (n=4). LDL was oxidized with for up to 24hr in the presence of 7.5 mM CuSO_4 and level of FAG measured.

Figure 5. Shows the generation of relative level (%) of oxidative species of LDL-cholesterol. LDL-cholesterol isolated from normal subjects (n=25) was oxidized for different durations in the presence of 7.5 mM CuSO_4 and different species analyzed using HPLC.

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