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Small dense LDL oxidation in hypertensives and diabetics and prediction of Metabolic Syndrome

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\textbf{KEYWORDS}
Metabolic Syndrome; Small dense LDL; Oxidation; Paraoxonase

\textbf{Summary}

\textbf{Aim:} The aim of this study is to compare the extent of small dense low-density lipoprotein (sdLDL) oxidation in diabetic and hypertensive patients and to investigate their correlation with Metabolic Syndrome as per NCEP ATP III criterion.

\textbf{Design and methods:} In the present study, 120 human subjects, men and women were selected randomly from the age group of 30–75 yrs for the screening of Metabolic Syndrome. Family history and prevalence of diabetes and hypertension in different age groups were recorded. Their waist circumference was measured and lipid profile was also determined. The sdLDL was isolated and oxidized in vitro. Attention was focused on the peak oxidation time of sdLDL in vitro and estimation of serum paraoxonase (PON-1) activity of all volunteers.

\textbf{Results:} Our results have indicated an overall increase in total cholesterol (TC), triglycerides (TGs), low-density lipoproteins (expressed as mg/dL) as well as mean waist circumference (expressed as cm) of the diabetic (242 ± 10.1, 218 ± 21.6, 140 ± 4.9, 105 ± 1.6) and hypertensive subjects (220 ± 6.4, 250 ± 12.9, 150 ± 5.6, 104 ± 1.9) as compared to controls (182 ± 5.4, 142 ± 8.9, 112 ± 4.3, 86 ± 1.5). It was also found that most of the diabetic and hypertensive subjects were from the age group of 50–59 yrs. The results obtained after oxidation of sdLDL have shown an early peak of oxidation in hypertensives [peak value = 65.3 ± 5.6 s] followed by diabetics [peak value = 75.7 ± 3.8 s]. Antioxidant enzyme paraoxonase was also found to be compromised in hypertensives and in diabetics.

\textbf{Conclusion:} The early peak of oxidation of sdLDL in hypertensives put them at a higher risk to CVD as compared to diabetics and abnormal lipid profile and increased waist circumference of diabetics and hypertensives suggest them to be potential patients of Metabolic Syndrome as per NCEP ATP III criterion.

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Introduction

The observation that certain risk factors accumulate in coronary patients is suggestive of a common origin and was the subject of quite early reports in clinical literature [1,2]. The concept has evolved through a number of synonyms [3] to its present status as the Metabolic Syndrome [4]. The strongest candidate for the common origin is insulin resistance [4,5]. A growing number of metabolic anomalies are linked to insulin resistance [3], the core components as excess weight, dyslipidemia and hypertension have recently been incorporated into guidelines by WHO [6] and National Cholesterol Education Programme Adult Treatment Panel III (NCEPATP III) [7] to provide a definition of the Syndrome which should aid clinical studies. Insulin resistance (or indications for abnormal glucose metabolism) is the central feature of WHO definition whereas the NCEP ATP III recommendations do not require evidence of insulin resistance.

Patients with the Metabolic Syndrome have significantly decreased low-density lipoprotein (LDL)—cholesterol/apo-A1 ratio which is related to the formation of small, dense lipoprotein particles that has higher susceptibility to oxidative modifications [8]. Hypertensive patients may have a preponderance of small dense LDL (sdLDL) particles; a phenomenon associated with an atherogenic lipoprotein profile and a 3-fold increased risk of cardiovascular diseases [9]. Additional risk factors that may be associated with the Syndrome include Type-II diabetes, hyperurecemia, microalbuminuria, and coagulation abnormalities that constitute a prothrombic diathesis [10]. Several studies have found an inverse relationship between the lag time of in vitro LDL oxidation and the severity and progression of coronary atherosclerosis [11], suggesting that enhanced susceptibility to oxidation may underlie the excess vascular disease observed in patients with diabetes.

The Syndrome is also associated with reduced concentrations and activities of the antioxidant enzyme paraoxonase-1 [8]. Human serum paraoxonase (PON-1), a 43 kDa protein catalyses the hydrolysis of organophosphate esters, aromatic carboxylic acid esters and carbamates in a calcium dependent manner [12]. Although the natural substrate for PON-1 is still unknown, several groups have reported that the enzyme protein has the capacity to retard the accumulation of lipid peroxides in low-density lipoprotein (LDL) and this is mainly due to the ability of the enzyme to reduce hydroperoxides [13]. Aviram et al. [14] have shown an inverse correlation between the esterolytic activity of PON-1 in serum susceptibility of HDL to oxidation. The Metabolic Syndrome is well characterised by the presence of smaller, denser lipoprotein particles that increase their susceptibility to oxidative modification and a diminished serum PON-1 which is a major determinant of the antioxidant capacity of HDL. These may be contributory factors to the increased presence and severity of coronary diseases in such patients [8].

We through the present study, therefore have examined the kinetics of in vitro oxidation of sdLDL after isolating it from the serum of volunteers. Attention was focused on the peak obtained for oxidized sdLDL. The arylesterase activity of PON-1 was also recorded in the respective volunteers.

Subjects and methods

A total of 120 subjects were recruited on a consecutive basis for a screening program to check the prevalence of Metabolic Syndrome at the J.N. Medical College, A.M.U. Men and women aged 30–75 yrs participated in this study. All the volunteers, unaware of their health status and not observing any medication schedule for either diabetes or hypertension, were randomly selected and divided into three categories: diabetics (those having fasting plasma glucose level ≥110 mg/dL but no hypertension), hypertensives (those having BP ≥130/80 mmHg but no diabetes) and controls (those having neither diabetes nor hypertension). Demographic information and clinical history was obtained after getting informed consent. Waist circumference of all volunteers was measured using standard techniques. Fasting blood specimens were obtained for determination of plasma glucose, serum cholesterol, serum triglycerides (TGs), serum HDL and LDL cholesterol.

Plasma glucose was determined by the glucose oxidase peroxidase method [15] and the concentration of serum cholesterol, serum triglycerides, and HDL cholesterol were measured by an enzymatic colorimetric test using commercial kits from Ranbaxy Diagnostic Division, HP. LDL cholesterol was calculated using the Friedewald equation [16].

Small dense LDL-C assay

The details and validation of this experiment have been described elsewhere [17]. In brief, the precipitation reagent (0.1 mL) containing 150 U/mL of heparin sodium salt and 90 mmol/L MgCl₂ was added to a serum sample (0.1 mL) and the samples were incubated for 10 min at 37 °C after mixing. Then each sample was placed in an ice-bath and allowed to stand for 15 min after which the precipitates were collected by centrifugation at 15,000 rpm for 15 min at 4 °C. The precipitates were always
packed tightly at the bottom of the tube and the supernatant was clear. This heparin-Mg\(^{2+}\) supernatant contains sdLDL cholesterol with no influence of other lipoproteins [18]. Nitrogen gas was bubbled through the supernatant collected in separate tubes which are then closed tightly with parafilm and kept at 4 °C in dark to protect it from oxidative modification.

**Oxidation of small dense LDL**

The procedure for oxidation of small dense LDL was adapted from the method described by Esterbauer et al. [19] for LDL oxidation. 10 |x|L of supernatant containing sdLDL, collected above, oxygenated PBS and 32 |x|L of 1 mM CuCl\(_2\) was added to a 2 mL quartz cuvette and the oxidation reaction products, the conjugated dienes, were monitored continuously at 37 °C on a UV spectrophotometer at 234 nm [20].

**Measurement of arylesterase activity of PON-1 using phenylacetate as a substrate**

Initial rates of hydrolysis were determined spectrophotometrically at 270 nm in a power wave 200 microtitre scanning spectrophotometer. The assays were performed in a final volume of 250 |x|L containing 1 mmol/L phenylacetate and 2 mmol/L CaCl\(_2\) in 20 mmol/L Tris–HCl buffer, pH 8.0 in the presence of 0.1 |x|L of serum. The extinction coefficient at 270 nm for the reaction was 1307 mol/L/cm. One unit of arylesterase activity is equal to 1 |x|L of phenylacetate hydrolysed per milliliter per minute [21].

**Statistical analysis**

Analysis was performed using origin 6.1 statistical package for windows. Continuous variables were expressed as mean ± S.D. and qualitative data were expressed in percentages. Lipid profile, waist circumference, oxidation of sdLDL and arylesterase activity of PON-1 were compared by Student’s t-test. P-value less than 0.05 were considered to be significant.

**Results**

A total of 120 subjects were included in the study and were divided into three categories, i.e. diabetics, hypertensives and controls (those having neither diabetes nor hypertension). Fig. 1 shows that among them 30% were diabetic, 13% were hypertensives and 57% were controls (as per their fasting plasma glucose and BP).
Fig. 3 Family history of (a) diabetic and (b) hypertensive subjects. Results are expressed as n(%).

Fig. 4 shows the age related prevalence of diabetic and hypertensive subjects. It is indicated in both Fig. 4(a and b) that the prevalence of diabetes and hypertension increased with age and the maximum prevalence was seen in the age group of 50–59 yrs.

The lipid profile of diabetic, hypertensive and control subjects is shown by determining their total cholesterol (TC), TGs, HDL and LDL (Fig. 5(a–d)). The average cholesterol of diabetics (242 ± 10.1) was found to be maximum followed by hypertensives (220 ± 6.4) and controls (182 ± 5.4). Mean triglycerides were highest in hypertensives (250 ± 12.9), then in diabetics (218 ± 21.6) and lowest in controls (145 ± 8.9). HDL was almost stable in all cases (diabetics = 45 ± 1.1, hypertensives = 44.3 ± 1.4 and controls = 40 ± 1.5).

Fig. 5 (a) Total cholesterol levels in diabetic, hypertensive and control subjects. Results are mean ± S.E.M. P < 0.05, using paired t-test. (b) Triglycerides levels in diabetic, hypertensive and control subjects. TGs >150 mg/dL defines Metabolic Syndrome (NCEP ATP III guidelines). Results are mean ± S.E.M. P < 0.05, using paired t-test. (c) HDL levels in diabetic, hypertensive and control subjects. Results are mean ± S.E.M. P < 0.05, using paired t-test. (d) LDL levels in diabetic, hypertensive and control subjects. Results are mean ± S.E.M. P < 0.05, using paired t-test.
Table 1: Average peak value of oxidation of small dense LDL (sdLDL) of diabetic, hypertensive and control subjects

<table>
<thead>
<tr>
<th></th>
<th>Peak (s)</th>
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<tbody>
<tr>
<td>Hypertensives</td>
<td>65.3 ± 5.6</td>
</tr>
<tr>
<td>Diabetics</td>
<td>75.7 ± 3.8</td>
</tr>
<tr>
<td>Controls</td>
<td>86.5 ± 4.1</td>
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Results are mean ± S.E.M. Early peak of oxidation of sdLDL of hypertensives shows its greater susceptibility to oxidation than followed by diabetics and controls.

controls = 35 ± 0.95) while LDL was highest in hypertensives (150 ± 5.6), followed by diabetics (140 ± 4.9) and controls (112 ± 4.3).

Recently sdLDL has been highlighted as a new risk factor for coronary heart diseases (CHD). It was discovered that the combination of Heparin and Magnesium precipitated a part of LDL which remained in the supernatant and was identified as sdLDL which was identical to the one isolated by ultracentrifugation. This supernatant was used as a source of sdLDL in the present study. It was oxidized in vitro by using CuCl₂ as described in Section ‘Subjects and methods’ [19] and the kinetics was observed in terms of peak time. Our results indicate the earliest peak of oxidation of sdLDL for hypertensive patients followed by diabetic and control subjects (Table 1).

The HDL-associated enzyme PON-1 has been identified as an important determinant of the capacity of HDL to prevent oxidative modification to LDL [14] and the activity of this enzyme has also been reported to be decreased in cardiovascular diseases (CVD) [22] and in diabetes [23]. Therefore we have also measured the arylesterase activity of PON-1 in all the three categories (Fig. 6). Our results have shown that the PON-1 activity was significantly reduced in case of hypertensives (0.26 ± 0.18) followed by diabetics (0.39 ± 0.14) and then by control subjects (0.60 ± 0.14).

Discussion

The clustering of multiple metabolic abnormalities including obesity, insulin resistance, non-insulin dependent diabetes mellitus (NIDDM), hypertension and dyslipidemias have become known as the Insulin Resistance Syndrome or Multiple Metabolic Syndrome [24]. An elevation in blood pressure is one of the components of Metabolic Syndrome [25] and high blood pressure is a major risk factor for cardiovascular diseases [26]. Therefore, the increased risk of cardiovascular diseases in hypertensive patients correlates with blood pressure and may be related to other factors like abnormal lipid profile [27]. Studies have also shown an association between hypertension and the oxidation of LDL and particularly the fact that its susceptibility to oxidation is greater in patients with essential hypertension than in normotensive subjects [28,9]. It is suggested that oxidative modification of LDL could promote and accelerate the development of atherosclerosis [29,30]. When LDL is chemically modified, an uncontrolled uptake of oxidized LDL by scavenger receptors in macrophages occurs. As a consequence, they dedifferentiate into foam cells that accumulate in the arterial wall, forming early sclerotic lesions [31].

Oxidation of LDL is a process of lipid peroxidation. The polyunsaturated fatty acids of LDL are successively degraded to different products [32]. The oxidative susceptibility of LDL is increased when combined with cardiovascular risk factors like the abnormal lipid profile [33]. It has been observed that patient with hypertension has a significantly lowered lipid peroxidation lag time compared to normotensive subjects [9]. There is evidence of a genetic influence on the LDL sub-fraction pattern and possibly the atherogenic potential [34]. Previously it has been reported that the sub-fraction of LDL, namely dense LDL, light LDL and very light LDL have different susceptibility for lipid peroxidation in vitro [35] The results of the present study therefore focuses on the sub-fraction of LDL, that is sdLDL as the literature points towards the sdLDL being particularly atherogenic [36]. Hypertensive patients have a preponderance of sdLDL particles, a phenomenon associated with atherogenic lipoprotein profile and a three-fold increased risk of cardiovascular disease [9]. This may probably be due to the high susceptibility of sdLDL to oxidation. We have demonstrated that the peak time of oxidation of sdLDL was earliest in hypertensives as compared to controls.
Surprisingly, in diabetics the peak time was much delayed as compared to that in hypertensives. This probably was due to the fact that very high concentration of glucose in blood may act as an antioxidant [37] as there is precedence in literature for the “antioxidant” effects of glucose and other sugars [38] and molar concentration of mannitol has long been used as a radical scavenger [39]. Lower concentration of glucose may facilitate or sustain oxygen radical production whereas very high concentration might be related to the ability of glucose to generate advanced glycation end products (AGE), or to initiate oxygen radical production [40]. The delayed peak of oxidation in diabetes may not be suggestive of reduced risk of cardiovascular diseases as the lipid profile of diabetics remains abnormal with serum cholesterol, LDL and TGs elevated as in hypertensives. This could only be suggestive of elevated glucose acting as antioxidant and probably leading to formation of advanced glycation end products.

HDL have been shown to potentially reduce oxidative modifications of LDL. The prevention of lipoperoxide generation during copper-induced LDL oxidation by HDL could be due to their enzyme content, such as paraoxonase-1 (PON-1). The exact mechanism by which PON-1 exerts its protective effect is not well established. It has been proposed that the antioxidant effect could be associated with the peroxidase-like activity of this enzyme. Thus, by hydrolysing preformed lipid peroxides, PON-1 can delay or inhibit the initiation of oxidation induced by metal ions [41]. Our results have shown that the serum levels of the enzyme are reduced in patients with hypertension and diabetes being much reduced in hypertensives. Thus, in hypertensives, more qualitative changes occur to LDL which render them more susceptible to oxidation; coupled to a reduction in the potential antioxidant activity of HDL. This is suggestive of hypertensives as high-risk group for CVD as compared to diabetic patients. Moreover, the TGs, LDL cholesterol as well as waist circumference in diabetic and hypertensive subjects remained elevated. So these are viewed as a severe form of Metabolic Syndrome patients. Further studies are however needed to conclusively suggest the relation between oxidation of sdLDL and increased risk of CVD.

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References


