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Human Serum Paraoxonase Activity in Patients with Type II Diabetes Mellitus as Compared to that of Control Subjects

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Abstract: Diabetes is closely related to the development of coronary artery disease (CAD) and myocardial infarction (MI). The oxidative modification of low-density lipoprotein (LDL) in the artery wall is currently believed to be central to the pathogenesis of atherosclerosis. Oxidized LDL has also been shown to be cytotoxic to capillary endothelial cells. The inverse relationship between serum PON activity and the risk for atherosclerotic diseases suggests that PON hydrolytic activity on oxidized LDL may be related to its antiatherogenicity. Serum PON activity is decreased in subjects who have had a myocardial infarction and in subjects with type I and type II diabetes. In this study human serum paraoxonase activity in patients with type II diabetes mellitus were compared to that of control subjects. 100 patients and 30 controls were taken. The result of this study revealed that the PON activity of the patients of type 2 diabetes mellitus was lower than that of the controls. As PON is known to be an antioxidant, the decreased PON activity could be responsible for increased levels of ox-LDL in these patients with consequent development of diseases like myocardial infarction and hypertension.

Keywords: Coronary artery disease,Diabetes, Human serum paraoxonase, Hyperlipidemia, Hypertension, Myocardial Infarction, Low-Density Lipoproteins.

INTRODUCTION

Diabetes, hyperlipidemia, hypertension, and insulin resistance are closely related to the development of coronary artery disease (CAD) and myocardial infarction (MI).The abnormal lipids in such scenarios may be very crucial in the genesis of resulting outcome. The levels of lipoproteins such as LDL and HDL cholesterol may not be abnormal in patients with diabetes; the lipoproteins may be glycated, resulting in an abnormal function. Therefore the genes involved in lipoprotein metabolism and modification may be especially important in the development of CAD and MI in patients with diabetes.

Coronary artery disease, diabetes mellitus, dyslipidemia, and paraoxonase activity are closely related. The oxidative modification of low-density lipoprotein (LDL) in the artery wall is currently believed to be central to the pathogenesis of atherosclerosis. Oxidized LDL has also been shown to be cytotoxic to capillary endothelial cells. Therefore mechanisms that prevent the oxidation of LDL have received increasing attention in recent years. One such mechanism is prevention of LDL oxidation by high-density lipoprotein (HDL).

PON has received most attention because it is the enzyme present in the serum of mammals (as opposed to birds and insects), which is responsible for resistance to organophosphate toxicity. Human serum paraoxonase (PON1) is a calcium dependant esterase that hydrolyzes organophosphates such as paraoxon, diazoxon, sarin and soman, and also arylesters such as phenylacetate.

Studies suggest that dietary antioxidants may not be enough. Under certain conditions, the oxidative stress in the body is so great that it outpaces the activity of the antioxidants. Thus, the combination of preventing LDL oxidation by antioxidants with the breakdown of oxidized lipids by paraoxonase may be important in reducing oxidative stress and the resulting atherosclerosis.

Although the natural substrates for serum PON are unknown, recent studies suggest that PON prevents LDL oxidation by hydrolyzing lipid peroxides in the lipoprotein.

The inverse relationship between serum PON activity and the risk for atherosclerotic diseases suggests that PON hydrolytic activity on oxidized LDL may be related to its antiatherogenicity. Serum PON activity is decreased in subjects who have had a myocardial infarction and in subjects with type I and type II diabetes.

OBJECTIVES

It is felt that a detailed study of serum paraoxonase, in patients with established diabetes mellitus, could throw more light on the mechanism of increased peroxidative damage seen in this condition.

This study sought to analyze the following biochemical parameters using samples of blood drawn from patients with diabetes mellitus.

The following parameters were estimated

- Serum paraoxonase activity.
- Fasting blood glucose.
- The enzyme activity was studied in the patient and control groups using (I) $CaCl_2$ only and (ii) using $CaCl_2$ and NaCl.
- The paraoxonase activity was compared to the enzyme activity of the control group selected from healthy donors from Blood Bank of our hospital.
- Patients were 100 and controls 30.

MATERIALS AND METHODS

This study was conducted during a period of one year in our hospital.

- 100 patients and 30 controls were taken.
- The patients were based on the following inclusion and exclusion criteria:

Inclusion criteria

- Patients attending the Diabetic Clinic of our hospital formed the subjects of this study.
- Only adults with an established diagnosis of type II diabetes mellitus were included (known diabetics under treatment for more than 2 years)

Exclusion criteria

- Patients with type I diabetes mellitus were excluded.

Subjects

Selection of subjects for study was done as follows:

Controls

Controlswere healthy young adults, attending the blood bank of our hospital for voluntary blood donation.

Study groups

Were adult patients of type II diabetes mellitus. There were 100 patients ranging from 40 to 80 years of age.

Sampling

Patients reported to the lab after 12 hours of fasting. 5ml of blood samples were collected in Vacutainer$^{(R)}$ gel tubes and subjected to centrifugation. Clear serum was separated and used for the following biochemical investigations using standard clinical chemistry methods (analysis was carried out within an hour after the collection of samples):

- Fasting blood glucose
- Serum paraoxonase activity.

METHODS

These tests were carried out in auto-analyzer DADE-DIMENSION AR$^{(R)}$ clinical chemistry system.

Sl. No.	Parameters	Methods
1	Fasting Blood Glucose	Hexokinase-Glucose-6-phosphate dehydrogenase NADH method

Serum Paraoxonase (PON)

Serum paraoxonase activity was estimated by a spectrophotometric method using p-nitrophenyl acetate as the substrate.

Principle

The serum is incubated with buffered p-nitrophenyl acetate as a substrate. The rate of formation of p-nitrophenol was measured spectrophotometrically.

Reagents

Tris – HCl Buffer

To prepare 200mM solution, which then has to be diluted in 1 in 10 dilutions for use

Tris – 0.2 M is 24.2g/L

- 2.42 g/100ml – solution A.
- HCl – 0.2 M is 17.2 ml/ 1000ml.
- 1.72 ml in 100ml – solution B.

Stock Buffer

50ml of solution A & 26.8ml of solution B and the volume made upto 200ml.

Working Buffer

20m M/L – Dilute stock 1 in 10. This is used for diluting serum sample 1:1.

Tris Buffer containing 1mM $CaCl_2$.

Tris Buffer containing 1mM $CaCl_2$ + 1M NaCl.

Substrate
0.5 ml of alcohol containing 15mg of p-nitrophenyl acetate (Sigma Chemical Co.) freshly prepared.

Procedure
Non-enzymatic hydrolysis
3ml of buffer (maintained at 37^0C) taken in a cuvette and 50µl of substrate added to it. The rate of change of OD (optical density) was monitored for 2 minutes at 412nm.

PON activity
- ***In presence of $CaCl_2$:*** 3ml of buffer +50µl of serum (1:1 diluted) initial absorbance was adjusted to 0.500. 50µl of substrate was added & the rate of change of absorbance was recorded for 0', 30', 60', 90', 120' at 412nm.
- ***In presence of $CaCl_2$&NaCl:*** Done as previously described for $CaCl_2$.

Calculations
- PON activity was calculated as n moles of p-nitrophenol formed per minute per ml (volume of assay mixture).
- The molar absorptivity of p-nitrophenol
  = 17,000 / M/ cm
  ≡ 0.017 / n mol / cm / ml
  ≡0.00567 / n mol / 3ml
  (As total volume was 3ml)

So, enzyme activity in 50µl of serum under assay conditions = $\frac{\Delta OD/min}{0.00567}$

=ΔOD/minute X 176 µmol/ml/min

Final activityActivity expressed as n moles p-nitrophenol formed per minute per ml (volume of assay mixture).
[ΔOD is the corrected OD, i.e., Total OD–non-enzymatic hydrolysis (0.125)]

STATISTICAL ANALYSIS
This was carried out by making use of the student's t-test. The correlation coefficient was also done between the various parameters.

Observation & results
The major aim of the study was to investigate the PON activity in patients with type II diabetes mellitus as compared to that of control subjects.

The result of this study revealed that the PON activity of the patients was lower than that of the controls.

PON activity of patients with Type II diabetes mellitus and controls (Graph – I)

Parameters	Patients (n=100)*		Controls (n=30)*	
	With $CaCl_2$	With $CaCl_2$&NaCl	With $CaCl_2$	With $CaCl_2$&NaCl
PON Activity	42.28± 13.88	38.14± 15.08	59.68± 8.96	57.28± 13.23

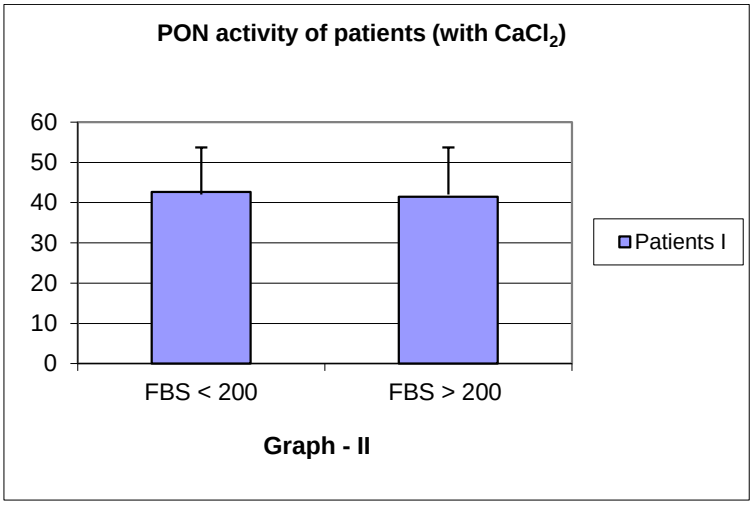
* n = Number of Observations

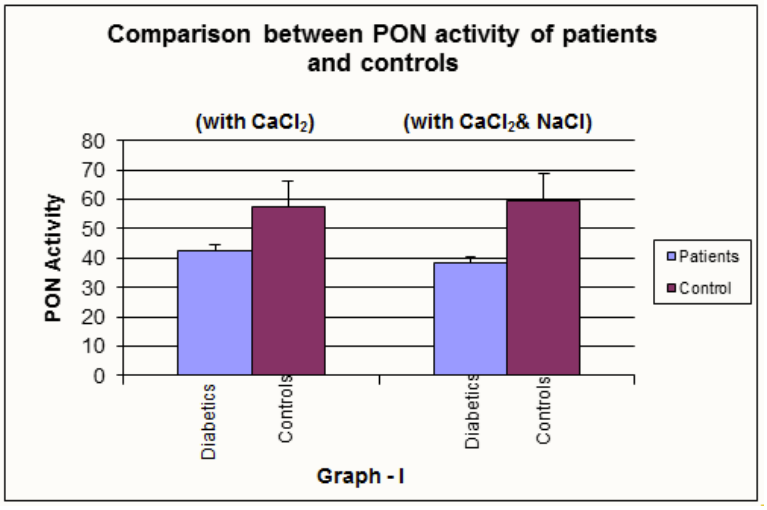
Parameters	Patients Vs Controls (with $CaCl_2$)	Patients Vs Controls (with $CaCl_2$&NaCl)
PON Activity	P < 0.001	p < 0.001

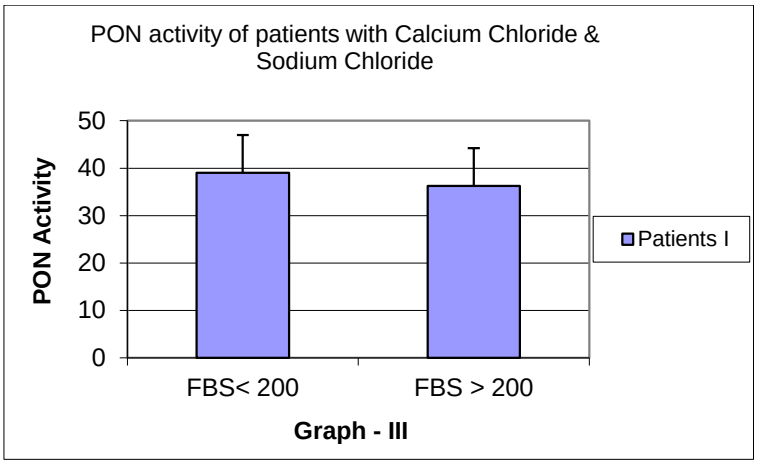
PON activity: In this study, the PON activity was found to be lower in the patients as compared to the controls. The values range from (with $CaCl_2$): 4.4 – 73.48, (with $CaCl_2$&NaCl): 5.10 – 71.19; p < 0.001. *The difference was highly significant.*

RESULTS

Parameter	Groups compared	Level of significance
PON activity With $CaCl_2$	Diabetics Vs controls	Highly significant
PON activity With $CaCl_2$&NaCl	Diabetics Vs controls	Highly significant







So, PON1 activity was found to be significantly lower in the serum of patients of type 2 diabetes mellitus compared to that of healthy controls.

DISCUSSION

Non-insulin-dependent-diabetes-mellitus or type II diabetes mellitus is the most common of the hyperglycemic states. It involves two defects, peripheral insulin resistance and hyperinsulinemia, in the prediabetic phase, which is followed by subsequent failure of insulin secretion to compensate for insulin resistance, with resultant hyperglycemia and overt diabetes[1]. The relation between alterations of lipid metabolism, obesity and diabetes has been recognized for decades [1]. Complications of diabetes have been classified as microvascular and macrovascular. Traditionally, retinopathy, neuropathy, and nephropathy have been designated as microvascular complications,

whereas atherosclerosis and its sequelae (stroke, myocardial infarction, and gangrene) are termed as macrovascular complications [1].

Diabetes is associated with late complications involving eyes, kidneys, nerves, and blood vessels. It is one of the leading causes of adult blindness and a major cause of renal failure, gangrene, and myocardial infarction and stroke [2]. The terms non-insulin dependent diabetes mellitus (NIDDM) and type II diabetes are usually used synonymously [3, 4].

Oxidation of LDL

Elevated LDL levels are thought to become atherogenic after oxidation to minimally modified LDL (LDL mm), which induces formation of monocyte chemotactic factor, endothelial monocyte receptor, and monocyte colony -stimulating factor. Uptake of oxidized LDL by monocytes / macrophages through the acetyl LDL (scavenger) receptor leads to the formation of the foam cells, the precursor of atherosclerotic lesion. AGEs play a role in triggering lipoprotein oxidation, and auto-antibodies against glycated LDL and glycated oxidized LDL are present in diabetic patients. The increased uptake of LDL by macrophages and fibroblasts in the presence of glucose is probably caused by lipid peroxidation through an oxidative pathway involving superoxide [5].

Therefore, the formation of oxidized LDL, which may participate in the pathogenesis of atherosclerosis in a number of ways, is an attractive solution to the LDL paradox [6].

Serum Paraoxonase: About the Enzyme

Human serum paraoxonase (PON1) is a calcium dependent esterase that hydrolyzes organophosphates such as paraoxon, diazoxon, sarin and soman, and also arylesters such as phenyl acetate [7].Although the natural substrates for serum PON are unknown, recent studies suggest that PON prevents LDL oxidation by hydrolyzing lipid peroxides in the lipoprotein [7]. The inverse relationship between serum PON activity and risk for atherosclerotic diseases suggests that PON hydrolytic activity on oxidized LDL may be related to its antiatherogenicity [7].

It was found that treatment of oxidized LDL with purified PON1 significantly reduced the ability of this lipoprotein to induce monocyte endothelial interactions, and that this effect was associated with a decrease in the oxidized phospholipid component in the LDL particle (particularly of oxidized 1-palmitoyl-2-arachydonyl-glycero-3-phosphoryl-choline) [7]. It was suggested that the physiologic function of PON1 might be to protect against the induction of inflammatory responses in arterial wall cells by destroying biologically active phopholipids in oxidized LDL [7]. It was later shown that PON1's free sulfhydryl group on the cysteine-284 residue is required for lipid peroxide destruction in LDL [8]and that the PONI molecule is partially inactivated in the process.

In this study it is seen that in the diabetic patients PON activity is decreased compared to controls but the control of blood sugar has no bearing on PON activity.

Preexisting low levels of PON may be present, thinking in the lines of "common soil" hypothesis discussed above. Low levels of plasma PON may be one of the components of the IRS. Level of PON1 in serum is likely to be under genetic control and thus is likely to be subject to genetic variation [9] or may be a consequence of DM. Probably at the genetic level these subjects will have low PON associated with dyslipidemia which is more common in diabetes. Hence in type II diabetic patients the functional role of paraoxonase is more important than in non-diabetic subjects [10]. Paraoxonase purified from native HDL is able to protect LDL from oxidative modification by destroying lipid peroxides. Thus this enzyme is implicated in the pathogenesis of atherosclerosis by protecting lipoproteins against peroxidation. Its biallelic gene polymorphism at codon 192 (glutamine/arginine) has been associated with CAD [9].

Chronic hyperglycemia coupled with a low PON activity causes considerable modification of protein structure and function due to non-enzymatic glycation of aminoacid residues. LDL cholesterol containing glycatedapolipoprotein B-100 interacts with vascular endothelium and decreasing thrombolyitc prostaglandins. In addition, glycated LDL cholesterol is more rapidly oxidized, resulting in accelerated macrophage uptake by the scavenger receptor pathway. It can therefore be speculated that the protective effects of paraoxonase against peroxidation of LDL particles are more important in diabetic patients. The difference could explain the much clearer effect of the paraoxonase 192 genotype on CAD in type II diabetic patients than in general populations [9].Besides its protective effects against LDL peroxidation, HDL-associated paraoxonase has recently been demonstrated to inhibit the oxidative damage of HDL as well [9]. This effect could be crucial in states that favour oxidation such as hyperglycemia or cigarette smoking, because the oxidation of HDL not only reduces its capability to prevent the oxidative modification of LDL, but also diminishes the ability of HDL to function as a potent acceptor for cholesterol efflux [9]. The lowered PON activities might impair the protective capability of HDL.

SUMMARY & CONCLUSION

- In this study serum paraoxonase activity has been studied in patients with type II diabetes mellitus and in healthy voluntary blood donors.

- Serum PON activity has been found to be significantly lower in diabetics as compared with normal subjects.
- Patients with diabetes mellitus are more susceptible to oxidative stress as a consequence of their chronic hyperglycemia.
- As PON is known to be an antioxidant, the decreased PON activity could be responsible for increased levels of ox-LDL in these patients which in turn leads to development of complications like coronary artery disease (CAD) and myocardial infarction (MI).
- To conclude, there are several interacting factors in diabetes like hyperglycemia, dyslipidemia and increased oxidative stress, superimposed on genetic predispositions which are responsible for the accelerated atherosclerotic changes that occur in these patients.

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