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OXIDATIVE STRESS PRODUCTS IN TYPE 2 DIABETES AND THEIR RELATION TO PARAOXONASE 1 ACTIVITY

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ABSTRACT
The present study indicated a significant increase in the serum advanced oxidation protein products (AOPP) and malondialdehyde (MDA) a marker of lipid peroxidation, in diabetic group compared to control group. AOPP was found to be positively correlated with MDA and Glycated haemoglobin [HbA_{1c}]. AOPP was negatively correlated with Paraoxonase- 1 (PON1) and this correlation was statistically not significant. PON1 activity was found to be decreased in diabetic group compared to control. Negative correlation was found between PON1activity and MDA, HbA_{1c} and with diabetic period. There was no correlation found between AOPP and diabetic period. On other hand, MDA level was positively correlated with HbA_{1c} and diabetic period. The studies also indicated that there is no difference in the level of AOPP, MDA and PON1 between vegetarian and non-vegetarian subjects. The negative correlation between PON1 level with the levels of MDA, AOPP, HbA_{1c}, and diabetic period indicates the role of PON 1 enzyme in oxidative stress. The positive correlation between the levels of MDA and AOPP supports the coupling process of lipid and protein oxidation

INTRODUCTION

Oxidative stress is an imbalance resulting from increased pro-oxidant generation and/or decreased anti-oxidant defenses. It is implicated in the pathogenesis of various diseases, such as atherosclerosis, diabetes, hemochromatosis, ischemic reperfusion injury, and rheumatoid arthritis, and in the normal aging process (Loeckie *et al.,* 1999). Increased oxidative stress in type 2 diabetes is a complex process which results from the abnormalities in the metabolic pathway. Hyperglycemia can increase oxidative stress through several pathways. Nishikawa *et al*. (2000) have shown that hyperglycemia induced overproduction of mitochondrial superoxide promotes the formation of advanced glycosylation end products, Protein kinase -C activation and hexosamine pathway activity. Lipid peroxidation induced by oxidants and oxidative stress, generates a huge variety of lipid peroxidation products, including reactive carbonyl compounds [RCCs] (Negre-Salvayre *et al.,* 2008). RCCs such as aldehydes and dicarbonyls, including hydroxyalkenals, acrolein, malondialdehyde (MDA), glyoxal and methylglyoxal, exhibit a large panel of biological properties. These aldehydes react on cellular and tissue proteins to form advanced lipid end products (ALEs) that induce protein dysfunctions and alter cellular responses (Petersen and Doorn, 2004). One of the best-known effects of ALEs precursors is their role in the modification of LDLs which is involved in the formation of early atherosclerotic lesions (Chisolm and Steinberg, 2000). For many years, lipid oxidation has been the focus of investigation, but due to their relatively high abundance, it is now recognized that proteins are the main targets for oxidation. The oxidation of protein plays a key role in the pathogenesis of degenerative and cancer diseases (Olas *et al.,* 2008). Advanced oxidation protein products (AOPPs) were first described by Witko-Sarsat *et al.,* (1996) and are formed during oxidative stress by the action of chlorinated oxidants, mainly hypochlorous acid and chloramines (Mcgarrigle *et al.,* 2007). AOPPs are defined as dityrosine containing cross linked protein products and are considered to be reliable markers to estimate the degree of oxidant mediated protein damage. These products originate as a result of the action of free radicals on proteins and may act as inflammatory mediators triggering the oxidative ignition of neutrophiles, monocytes, and T- lymphocytes, thus leading to upregulation and excessive stimulation of dendritic cells (Kalousova *et al.,* 2002).

Paraoxonase-1 (PON1) is a protein of 354 amino acids with a molecular mass of 43 kDa (Primo-Parma *et al.,* 1996). Human PON1 is synthesized in the liver and secreted into the blood and is associated exclusively with HDLs in the serum (Mackness, 1989). Human serum PON1 has been implicated in the detoxification of organophosphates, and possibly in the prevention of lipid peroxidation of low-density lipoprotein (Mackness *et al.,* 2000). It has been shown that human serum paraoxonase activity is a predictor of coronary artery disease [CAD] (Mackness *et al.,* 2003). PON1 reduces oxidized lipids in human atherosclerotic lesions derived from carotid or

coronary artery specimens (Aviram *et al.,* 2000). PON1 directly inhibits macrophage oxidative stress and therefore, decreasing the ability of macrophages to release superoxide anions and to oxidize LDL (Rozenberg *et al.,* 2003). Reduced paraoxonase activities have been reported in several groups of patients with diabetes, hypercholesterolemia and cardiovascular disease who are under increased oxidative stress (Mackness *et al.,* 1991; Ayub *et al.,* 1999). There are no reports on the AOPP and its relation with PON 1 and lipid peroxide in type 2 diabetes. Hence, the present study was undertaken to evaluate the AOPP, lipid peroxide marker and PON 1 activity in type2 diabetic patients as well as to study the relation between them and other biochemical markers.

MATERIALS AND METHODS

Study population

All the studies were carried out at The Department of Biochemistry, Bangalore University, Bangalore. The study population were identified and conducted in the KC General hospital, Bangalore. The study population included 120 subjects, out of them 60 are non-diabetic healthy control without any symptoms of acute inflammation and the other 60 are patients with type 2 diabetes mellitus with a mean disease duration of 3.91 ± 3.53 years. All the diabetic patients were on treatment with oral hypoglycemic agents. All patients were in a stable clinical condition without any symptoms of acute infection. All subject were questioned and informed about the aim of the study and their consent has been obtained. Ethical clearance for this study has been obtained from the ethical Committee of the Institution. The clinical and general biochemical characteristics of the study population are shown in Table 1. Blood was collected after an overnight fasting from the antecubital vein. 3mL blood was transferred to EDTA added tubes for estimation of HbA_{1c}. The serum for analysis was obtained by collecting blood in a plain vacutainer and centrifuged at 3000 rpm for 15 minutes after keeping for 30 min at room temperature. The sera so obtained was stored at –70ºC.

Biochemical tests

Glucose, lipids and lipoproteins were estimated using enzymatic-colorimetric kits (ERBA Diagnostic Mannheim GmbH). LDL cholesterol was calculated using Friedewald formula (Friedewald *et al.,* 1972). HbA_{1c} was estimated using ion exchange resin method (Coral Clinical System kit).

Estimation of AOPP

The concentration of AOPP in the serum samples was determined by the spectrophotometric method of Witko- Sarsat *et al.,* (1996). The method is based on the color reaction of AOPP with potassium iodide solution in an acidic environment. 100μL of biological material was diluted to 1.0 mL with phosphate buffered saline. Then, 50 μL of 1.16M potassium iodide solution followed by 100μL 10% acetic acid were added. The absorbance of the solution was measured immediately at 340 nm against a suitable blank. The concentrations of AOPP were express as chloramines-T units (μmol/L).

Estimation of PON1 activity

PON1 arylesterase activity was measured spectrophotometrically according to the method of Gan *et al.,* (1991) with a slight modification. The assay mixture contained 2 mM of phenylacetate in 50 mM Tris–HCl buffer (pH 8.0) containing 2 mM of calcium chloride. The reaction was started by the addition of serum and the increase in the absorbence was recorded at 270 nm. Blanks were included to correct for the spontaneous hydrolysis of phenylacetate. The concentration of phenol formed was calculated by using 1.310 M^{-1} cm^{-1} as the molar extinction coefficient of phenol. One unit of arylesterase activity is defined as the amount of enzyme required to release 1 μmole of phenol per minute. The total activity is expressed as μmol / mL.

Estimation of MDA

MDA in the serum samples was estimated using Buege and Aust's method (1978). Serum samples were mixed with thiobarbituric acid (TBA) reagent (0.375% TBA, 15%TCA in 0.25 N HCl) and boiled for 10 min. After cooling, 2.0 mL of 0.2 N NaOH was added and vortexed for 10 min. Then the samples were centrifuged and the absorbances of the supernatants were measured at 533 nm against a suitable blank. MDA concentration was calculated using a molar extinction coefficient of 156 M^{-1} cm^{-1} and expressed as nmol/ mL.

Statistical analysis

All analysis were performed by using statistical software SPSS. Independent-sample t-tests were performed in the analysis of biochemical data. Two tailed probability (P) value were calculated and a value of $p < 0.05$ was considered as statistically significant. The correlation coefficient was calculated using Bivariate correlation method.

RESULTS

The biochemical characteristics of healthy control and type 2 diabetes study subjects are given in Table 1. The plasma lipid profile of type 2 diabetic patients showed a significant elevated level of T.C., T.G., LDL-C ($p < 0.05$), while HDL-C appeared to be reduced ($p < 0.05$). The levels of AOPP, MDA and PON 1 activity in control and diabetic group is tabulated in Table 2. The marker of oxidative stress AOPP and MDA were significantly higher in the serum of type 2 diabetes compared to control ($p < 0.05$). On other hand, PON 1 level was found to be low in the patients with type 2 diabetes compared to control ($p < 0.05$). The correlation analysis data between AOPP, PON1, MDA, HbA_{1c} and diabetic period in patient with type 2 diabetes is summarized in Table 3. The results showed significant positive correlation between AOPP with MDA ($r = 0.306^*$) and HbA_{1c} ($r = 0.275^*$) whereas, there was no significant correlation found between AOPP with PON1 and diabetic period in the subjects with type 2 diabetes . PON 1 level showed a negative correlation with MDA ($r = -0.602^{**}$), HbA1c ($r = -0.602^{**}$) and with diabetic period ($r = -0.372^{**}$). The MDA level showed a positive correlation with HbA_{1c} ($r = -0.571^{**}$) and with diabetic period ($r = -0.372^{**}$). The correlation analysis data of diabetic group between AOPP and PON 1 is shown in Fig.1 and AOPP and MDA in Fig. 2. The data

Table 1: Clinical and Biochemical characteristics of the control and type 2 diabetic subjects

parameter	Control	Diabetic group	Significance
Total No.	60	60	
Age (Year)	49 ± 12	52 ± 10	p = 0.150
Gender(M/F)	28 / 32	29 / 31	
MBI (Kg/m^2)	26.3 ± 4.4	27.2 ± 3.5	p = 0.191
Blood pressure Diastolic Systolic	84.6 ± 12.1127.3 ± 16.8	85.5 ± 10.5130.6 ± 15.4	p = 0.688p = 0.259
FBS (mg/dL)	86.8 ± 12.4	123.9 ± 23.4	p < 0.05
Cholesterol (mg/dL)	202.38 ± 15.91	225.60 ± 32.49	p < 0.05
Triglyceride (mg/dL)	160.53 ± 25.76	196.91 ± 61.71	p < 0.05
LDL (mg/dL)	118.88 ± 17.87	140.83 ± 32.80	p < 0.05
HDL (mg/dL)	51.98 ± 7.08	44.78 ± 9.66	p < 0.05
HbA_{1c} (%)		7.91 ± 1.83	
Diabetic period (Year)		3.91 ± 3.53	

The result are expressed as mean ± SD

Table 2: Serum levels of AOPP, MDA and PON 1 in control and diabetic groups

Parameters	Control n = 60	Diabetic group n = 60	Significance
AOPP μmoL/L	65.95 ± 21.769	103.38 ± 33.49	p < 0.05
MDA nmoL/mL	2.56 ± 0.764	3.27 ± 1.097	p < 0.05
PON1 μmoL/mL	93.33 ± 29.108	69.03 ± 21.55	p < 0.05

The result are expressed as mean ± SD

Table 3: Correlation analysis of AOPP, PON1, MDA, HbA_{1c}, and diabetic period in the patients with type 2 diabetes

Parameters analyzed	r	p Value
AOPP / PON1	– 0.206	0.114
AOPP / MDA	0.306*	0.017
AOPP /diabetic period	0.233	0.073
AOPP / HbA_{1c}	0.275*	0.034
PON1 / MDA	–0.602**	0.000
PON1 / HbA_{1c}	–0.602**	0.000
PON1 / diabetic period	–0.509**	0.000
MDA / HbA_{1c}	0.571**	0.000
MDA / diabetic period	0.372**	0.003

**Correlation is significant at the 0.01 level (2-tailed); *Correlation is significant at the 0.05 level (2-tailed)

indicated a negative correlation between AOPP and PON 1 level and is not significant. However, when analysis was done between AOPP and MDA, there was a positive correlation which is significant. The present study found no significant difference in the level of AOPP, PON1and MDA of the study population when the data was analyzed according to the diet (Table 4).

DISCUSSION

Oxidative stress is thought to be increased in a system where the rate of free radical production is increased and/or the antioxidant mechanisms are impaired. In recent years, the oxidative stress-induced free radicals have been implicated in the pathology of type-1 and type-2 diabetic patients (Brownlee *et al.,* 1986; Ashour *et al.,* 1999). The present study is undertaken to assess the AOPPs and their possible relation with PON1 activity and lipid peroxide in type 2 diabetes patients. Hyperglycemia in diabetic patients can increase the levels of free radicals through glucose autooxidation. This leads to non-enzymatic post-translational glycation of proteins resulting from chemical reaction between glucose and primary amino groups of proteins and also through polyol pathway and protein kinase activation (Forbes *et al.,* 2004, Tsai *et al.,* 1994). Our study showed significant increase in protein oxidation and lipid peroxidation as reflected by increase in the plasma level of AOPP, a marker of protein oxidation and MDA a marker of lipid peroxidation. The increase in protein oxidation and lipid peroxidation in diabetics in the present study are in accordance with earlier findings that hyperglycemia induces overproduction of oxygen free radicals in diabetes (Nourooz-Zadeh *et al*., 1997; Vanizor *et al.,* 2001; Pasaoglu *et al.,* 2004). Witko-Sarsat *et al*. (1996) have reported an increase in the level of AOPP in hemodialyzed patients without differences between diabetics and non-diabetics. AOPP was reported to be elevated in patients with CAD and considered to be independent risk factor for CAD status (Kaneda *et al*., 2002). Moreover, lipoperoxidation of cellular components, a consequence of free-radical activity, is thought to play an important role in aging, atherosclerosis, and late complications of diabetes (Kesavulu *et al.,* 2001). The level of PON1, the anti-oxidative and anti-atherogenic enzyme was found to decrease in patients with type 2 diabetes compared to control ($p < 0.05$). The decreased level of PON1 activity in type 2 diabetes may result in part from increased HDL-C glycation underglycemic stress. It has been reported that increased glycation of HDL leads to the impairment of its anti-atherosclerotic properties (Hedrick *et al.,* 2000). *In vitro* glycation of HDL appears to partially inhibit PON1 (Feretti *et al.,* 2001). It also has been reported that PON 1 is heavily glycated *in vivo* in people with type 2 diabetes and those with coronary heart desease which appears to inhibit PON 1's ability to metabolize membrane lipid hydroperoxides (Mastorikou *et al.,* 2008). Serum PON 1 activity was also shown to be decreased in

Table 4: Comparison of serum levels of PON1, AOPP, and MDA in vegetarian and non-vegetarian subjects

Parameter	Vegetarian (n = 30)	Non-vegetarian (n = 90)	p value
PON1 μmoL/mL	76.30 ± 29.55	82.81 ± 27.80	0.276
AOPP μmoL/L	93.53 ± 32.41	81.71 ± 33.96	0.098
MDA nmoL/mL	3.00 ± 1.04	2.89 ± 1.00	0.632

The results are expressed as mean ± SD

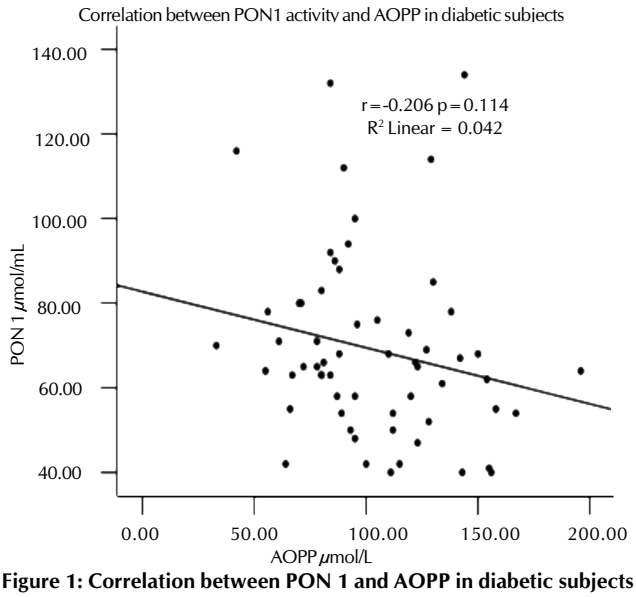

Figure 1: Correlation between PON 1 and AOPP in diabetic subjects

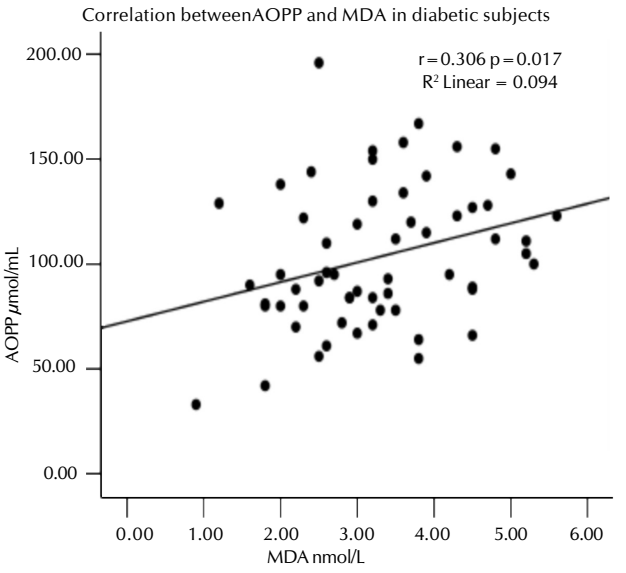

Figure 2: Correlation between AOPP and MDA in diabetic subjects

patients after myocardial infarction (McElveen *et al.,* 1986), in patients with familial hypercholesterolemia (Kaneda *et al.,* 2002), and in patients with diabetes mellitus (Mackness *et al.,* 1991) in comparison to healthy subjects. Our study also indicates that there is a significant positive correlation between AOPP with MDA (r= 0.306*) and HbA_{1c} (r = 0.257*) in the diabetic patient. This correlation seems to indicate the role of hyperglycemic inducing lipid peroxidation and protein oxidation. Many reports have appeared supporting the idea that protein and lipid oxidation are coupled processes. On the other hand, the interaction between lipids and proteins during oxidation is not well understood. The relationship between AOPPs and PON1 activity is not completely understood. In the present study, the correlation between AOPPs and PON1 activity in type 2 diabetic subjects was not found to be statistically significant (r = -0.206 p=0.114). In contrast, PON1 activity was found to be negatively correlated with MDA and HbA_{1c} (r= -0.602 p=0.000). PON1 was also significantly correlated with diabetic duration (r= -0.509 p=0.000). The significant elevation of MDA in type 2 diabetic patient indicates an increase in the lipid peroxidation. MDA reacts with Lys residues by forming Schiff bases and plays a major role in low-density lipoprotein (LDL) modification and their metabolic deviation towards macrophages (Raziel *et al.,* 2001). Oxidation of LDL lipids is thought to render the lipoprotein atherogenic, because oxidized LDL is more readily taken up by macrophages via scavenger receptors. Paraoxonase is an esterase residing on high-density lipoprotein. The antioxidant activity of HDL is largely due to PON1. The enzyme has found to protect both HDL and low-density lipoproteins (LDL) against peroxidation, which suggests a possible involvement of PON1 in the anti-atherogenic properties of HDL. It has been shown that in vitro purified paraoxonase decreased LDL lipid peroxidation (Durrington *et al.,* 1991) and that purified paraoxonase significantly reduced the ability of mildly oxidized LDL to induce monocyte endothelial interactions. All these findings substantiates the negative correlation between PON1 with MDA and oxidative stress. The oxidative stress induced through many pathways are associated with decrease of PON1 activity in type 2 diabetes as well as increase in the severity of diabetic complications . Inactivation of PON1 itself may lead to a predisposition to atherosclerosis as well as increased markers of oxidative stress. The increase in the level of T.C., T.G., LDL (p=0.000) with a decrease in the level of HDL observed in the present study are also implicated in the oxidative stress and pathogenesis of the diabetes mellitus.

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