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Decreased basal activity of HDL associated enzyme: Paraoxonase (PON) during uncompensated oxidative stress among type 2 diabetes mellitus patients

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Abstract The inverse relationship between serum levels of High Density Lipoprotein (HDL) and the development of Cardio Vascular Disease (CVD) risk among diabetic patients was known for several decades. Besides the decreasing quantity of HDL, the qualitative functions of HDL are adversely affected during uncompensated oxidative stress among diabetics and leads to implication of several complications such as dyslipidemia, lipid peroxidation, endothelial dysfunction and atherosclerosis. Therefore we have undertaken this study to determine anti-atherogenic property of HDL by measuring it's one of the associated enzymes; paraoxonase (PON) among type 2 diabetes patients, along with the serum activity of superoxide dismutase (SOD) as an index of antioxidant status and lipid peroxidation end product, i.e malondialdehyde (MDA) as a marker for oxidative stress. This study included a total of 56 untreated type 2 diabetic patients and 29 healthy volunteers as controls. FBS, PPBS, HbA_{1C} and fasting lipid profile were measured in both the study groups. Activity of basal PON, SOD and plasma MDA levels was determined in both the study groups according to standard clinical laboratory procedures. All the diabetic patients were under poor glycemic control. Serum levels of HDL between the two study groups are not significantly differed. But, serum basal PON and SOD activity were significantly decreased, whereas MDA levels were highly elevated (284±59 nM/mL/min, 111±35 μmol/L, 10.38±4.17 IU/mL respectively) when compared with healthy controls (371±46 nM/mL/min, 63±12 μmol/L, 16.91±2.89 IU/mL respectively). Although there is no significant reduction in concentrations of HDL in diabetics when compared with controls, but there was a significant decrease in anti-atherogenic property i.e. activity of paraoxonase enzyme. Moreover the serum activity of paraoxonase was significant and negatively correlated with MDA levels (r=- 0.53, P<0.001) as well as with FBS (r=- 0.30, P<0.05). Therefore the qualitative functions of HDL are significantly affected by hyperglycemia induced oxidative stress. Hence, we concluded that the quality of HDL is most important in order to determine oxidative stress related complications in diabetes mellitus than its concentration.

Keywords HDL · Paraoxonase · Glucose auto oxidation · Oxidative stress · Type 2 diabetes mellitus · CVD

Introduction

Diabetes is directly responsible for 3.5 % of Non Communicable Deaths (NCD) every year [1]. Oxidative stress is encountered in diabetes and contributes to various complications [2]. In vivo, production of free radicals and their neutralization is part of normal metabolism, but their levels were not compensated during conditions, such as diabetes [3]. This imbalance happens due to either changes in quantity or quality of antioxidants or increase in free radical production or both. Poor glycemic control is the principle metabolic disturbance in the implication of reactive oxygen species (ROS) production. Hyperglycemia results in overproduction of oxygen free radicals

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through auto oxidation of glucose, which contributes to the development of lipid peroxidation and atherosclerosis [4]. HDL was considered as good cholesterol and anti-atherogenic due to capable of reverse cholesterol transport (RCT) [5]. From the Framingham Heart Study it was concluded that, each 0.26 mM/L increase in HDL-C was associated with decrease in relative risk for CVD mortality [6]. However cholesterol efflux is not only the function of HDL. It is involved in several functions due to its antioxidant, anti-inflammatory, and anti-thrombotic effects [7]. It is well known that, HDL may have a protective role against oxidation of LDL [8–11]. Approximately 56 proteins are associated with it, such as apolipoproteins, lipid transfer proteins, proteins of hemostasis (platelet activating factor acetylhydrolase) and thrombosis, immune & complement, growth factors, receptors, antiatherogenic proteins such as paraoxonase, LCAT, platelet activating factor acyl-hydrolase (PAF-AH) etc. All these proteins are freely exchangeable during HDL metabolism [12]. In an observational study oxidation of LDL was reduced when serum samples were incubated with apoA1, LCAT and PON in the presence or absence of HDL. Therefore HDL associated proteins plays a key role in the diverse role of HDL [13]. Moreover, HDL is deficient of the heparin binding site; hence it does not stick to blood vessel walls unlike LDL. Hence it can enter into all extracellular spaces and involve in the transport of lipids and proteins. All these may contribute to its cardiovascular protective effects. Several studies showed that, all these HDL associated proteins are distorted during various pathological conditions such as hyperglycemia [14]; oxidative stress [15, 16], inflammation [17] and HDL become dysfunctional.

Serum Paraoxonase (PON) is a Ca^{2+} dependent aryl esterase (aryl dialkyl phosphatase, EC 3.1.8.1) synthesized and secreted by the liver [18, 19]. It is associated with apo A-1, a major lipoprotein of the high density lipoprotein complex that hydrolyzes organophosphate compounds, aryl esters, oxidized phospholipids and lipid peroxides. It protects both HDL and LDL against peroxidation, which suggests a possible involvement of PON in the anti atherogenic properties of HDL [20, 21]. Several studies found that the activity of PON is decreasing during oxidative stress, acute myocardial infarction [22], familial hypercholesterolemia, and in diabetes mellitus [23]. Thus, low serum PON activity has been implicated in the development of coronary heart diseases in diabetes.

Superoxide dismutase is a primary extracellular antioxidant enzyme, which is bound to heparin sulfate proteoglycans in vascular beds [24, 25]. It is involved in the direct elimination of reactive oxygen species such as superoxide radicals before they convert into most toxic radicals [26]. Serum levels of SOD were depleted when there is an overproduction of ROS [27].

Therefore we determined anti-atherogenic property of HDL in hyperglycemia induced oxidative stress among type 2 diabetes mellitus patients.

Materials & methods

We have included 56 untreated Type 2 DM (48 men & 8 women, mean age 58±11) who attended to the Diabetic OPD at PSG Hospital, Coimbatore without evidence of CVD. We also included 29 healthy staff-volunteers from the same institution. Both the patients and controls were non-smokers and non-alcoholics. Informed consent was obtained from both patients and healthy volunteers. This study was approved by Department of Clinical Research and Bioethics.

Sample collection

Venous blood samples were collected after 12 h fasting using Becton Dickinson vacutainers. Biochemical analysis was carried out on the same day of sample collection according to standard clinical chemistry laboratory procedures.

Procedures

Diabetic profile

Fasting and post-prandial plasma glucose and lipid profile were assessed on the Integra 400 plus fully automated analyzer with standard Roche kits according to clinical chemistry laboratory procedure.

HbA_{1C}

Glycosylated hemoglobin (HbA_{1C}) was measured on Integra 400 plus by an immunoturbidimetry method. Briefly, anticoagulated blood was hemolysed and total Hb measured colorimetrically based on the formation of a brownish-green chromophore (alkaline hematin) in alkaline detergent solution, which is proportional to the Hb concentration by monitoring the increase in absorbance at 552 nm. HbA1c is determined immunoturbidimetrically. The ratio of both concentrations yields the final percentage of HbA1c (HbA_{1C}%) [28, 29].

Serum basal paroxonase (BPON) activity

The activity of BPON was measured spectrophotometrically at 25 ° C by adding 50 μL of serum to 3 mL of tris buffer (20 mM). The initial absorbance was adjusted to 0.5 in spectrometer at a wavelength of 412 nm. The reaction was initiated by adding 50 μL of 5.5 mmoL p-nitrophenyl acetate as substrate. The rate of increase in absorbance (A) was

Table 1 Showing the demographic variables of two study groups

Demographic Variable	Type 2 DM	Controls
Males	48	18
Females	8	11
Age (Mean±2SD)	58±11	42±6
Total (n)	56	29

monitored for 2.5 min. The same protocol was followed in order to determine the rate of non-enzymatic hydrolysis [30].

The corrected 'A' was obtained by subtracting the non-enzymatic ΔA from the total ΔA.

PON activity was calculated by using molar extinction coefficient of 17,000 M^{-1} cm^{-1}

Lipid peroxidation end product- malondialdehyde (MDA)

TBARS as a measure of lipid peroxide (Malondialdehyde) was measured spectrophotometrically by using the method described by Draper and Hadley (1990). Briefly, 2.5 mL of 10 % trichloro acetic acid and 0.5 mL of plasma were added and mixed. After incubating for 15 min at 90 °C and cooling with cold water, the mixture was centrifuged at 3,000 rpm for 10 min. 2 mL of supernatant was taken and 1 mL of 0.675 % TBA was added. The tubes were sealed and incubated at 90 °C for15 min and then cooled to room temperature. The optical density was measured at 532 nm by a spectrophotometer. Concentrations of MDA were expressed in μmol/L [31].

Superoxide dismutase (SOD)

SOD activity was determined by the method of Marklund and Marklund (1974). In brief, superoxide anion is involved in auto-oxidation of pyrogallol at alkaline pH (pH-8.5). The Superoxide Dismutase (SOD) inhibits auto-oxidation of pyrogallol, which can be determined at 420 nm by a spectrophotometer. One unit of SOD is defined as the amount of enzyme required to cause 50 % inhibition of pyrogallol auto oxidation. The activity of SOD expressed in IU/mL [32].

Statistical methods

The difference between two study groups was performed by the independent student—t test (unpaired, parametric analysis). The Karl Pearson correlation coefficients were calculated to examine the correlation between various parameters. The normality distribution test was run for all the needed parameters. A multiple linear regression analysis was performed by considering MDA, PON and SOD as dependent variables. The possible associations between these dependent variables and independent variables (Age, FBS) were followed. All these statistical analysis was done using MedCalc—version 12.7.0. The data were expressed as mean±SD. The probability value<0.05 was considered as statistically significant.

Results

The demographic variables of two study groups are shown in Table 1. We tested the normality of distribution for various parameters (Table 3). All the parameters in both the study groups were accepted by the Kolmogorov-Smirnov normal distribution test except FBS, PPBS and HbA_{1C} in the case group. Age was not matched to compare the two study groups. We also performed multiple linear regression analysis for dependent (MDA, PON, & SOD) and independent variables (Age & FBS) were demonstrated in Table 2 and Figs. 1, 2, 3, 4, 5 and 6. From the regression analysis it was confirmed that 'age' was not a significant confounder for any of these dependant parameters in both the groups except SOD, which has been falling in response to increasing in age among controls. Whereas blood glucose is proved as a significant determinant factor for the increase in MDA levels and decrease in PON activity (Table 2).

The mean and SD of various parameters in both the study groups are shown in Table 3 & Fig. 7. Plasma MDA

Table 2 Showing the multiple linear regression analysis for Age, FBS and dependant variables

Variables	R^2		SE		T		P	
	Case	Control	Case	Control	Case	Control	Case	Control
Age Vs MDA	0.0047	0.0028	0.448	0.364	0.496	0.278	0.62	0.78
FBS Vs MDA	0.2935	0.0186	0.0727	0.184	4.6029	0.5478	<0.0001*	0.588
Age Vs PON	0.0352	0.0637	0.761	1.383	−1.38	1.355	0.174	0.186
FBS Vs PON	0.084	0.022	0.087	0.666	−2.224	0.72	0.03*	0.478
Age Vs SOD	0.0062	0.1393	0.054	0.0826	−0.58	−2.09	0.56	0.04*
FBS Vs SOD	0.00072	0.0005	0.0086	0.039	0.198	−0.12	0.84	0.904

(R^2—Coefficient of determination, SE- Standard Error, t—t—test, P—Probability)

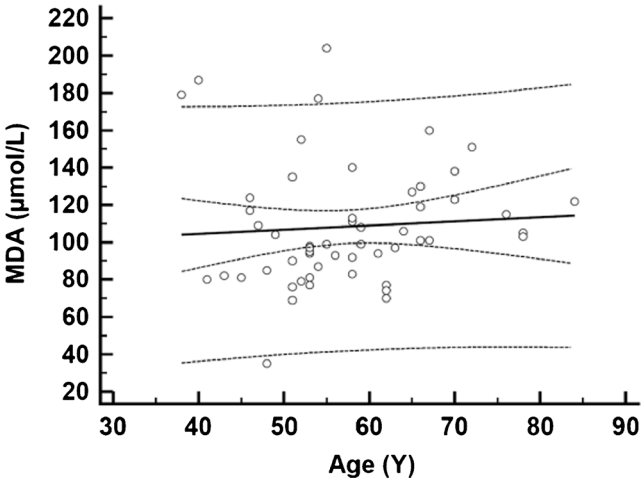

Fig. 1 Showing the scatter diagram with regression line for age and MDA in case group

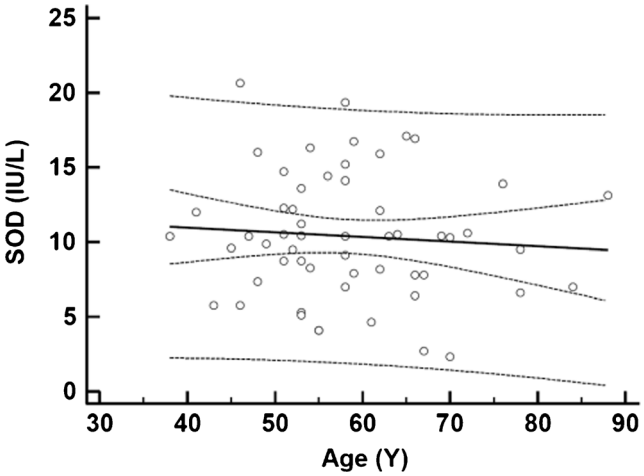

Fig 3 Showing the scatter diagram with regression line for age and SOD case group

levels were significantly increased (111±35 µmol/L) whereas serum activity of PON (284±59 nM/mL/min) and SOD (10.38±4.17 IU/mL) were significantly decreased in patient when compared with controls (63± 12 µmol/L; 371±46 nM/mL/min &16. 91±2.89 IU/mL respectively). The multiple linear regression analysis for various parameters were shown in Table 2. Age and FBS were considered as independent variables on outcome variables such as MDA, PON and SOD. Plasma MDA levels are independently elevated with age (coefficient of determination (R^2)=0.0047, P=0.62), but these levels are significantly dependent on glycemic control (R^2=0.2935, P<0.0001) as shown in Table 2. The same pattern was also observed for age on PON activity (Table 2). From regression analysis it was confirmed that age was not confounded to modify the outcome variables in both the study groups except for SOD, which is falling in increasing in age (R^2=−2.09, P<0.04) among controls not in the case group (R^2=−0.58, P=0.56). Among the lipid profile parameters, triacylglycerides (TAG) in patients was significantly elevated (153±89 mg %), but HDL and LDL cholesterol levels were not significantly differed between the groups. The ratio of HDL to PON in patients is significantly increased (0.14±0.05) than control group (0.1±0.026). The increased HDL to PON ratio in patients explains the decrease in PON activity, again which was not dependent upon the concentration of HDL. Moreover, HDL: PON ratio was significant and positively correlated with plasma MDA levels (r^2=0.48, P<0.01) whereas it was reversed in the control group (r^2=− 0.40, P<0.01).

The correlation coefficients of various parameters were shown in Table 4. Age was not significantly correlated with MDA levels and PON and SOD activity in both groups except SOD in the control group. There was a significant negative correlation existed between serum PON activity and MDA levels (r^2=− 0.53; P<0.01) as well as between MDA and

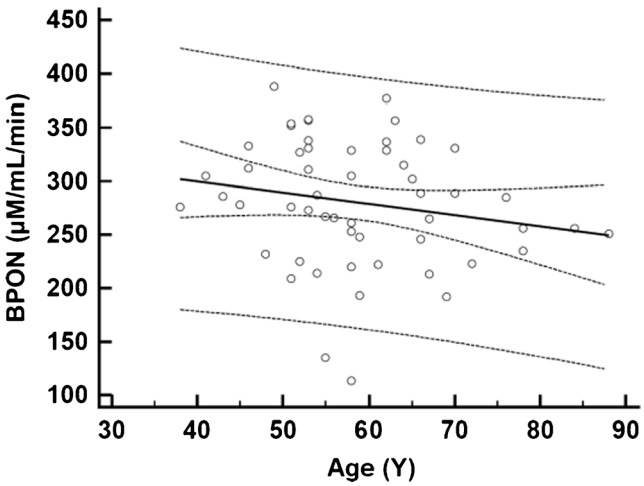

Fig 2 Showing the scatter diagram with regression line for age and BPON case group

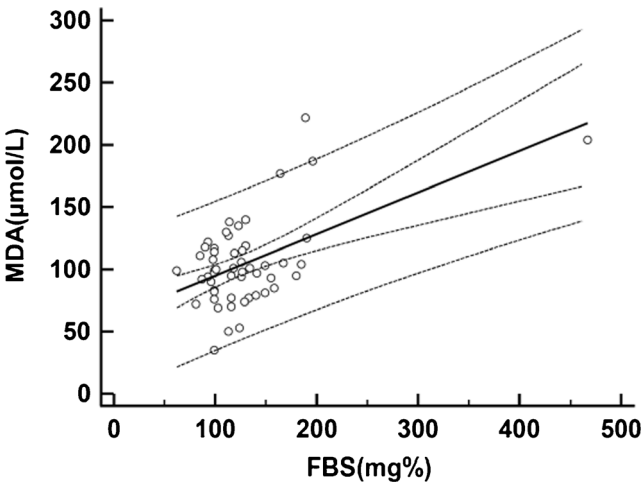

Fig 4 Showing the scatter diagram with regression line for FBS and MDA in case group

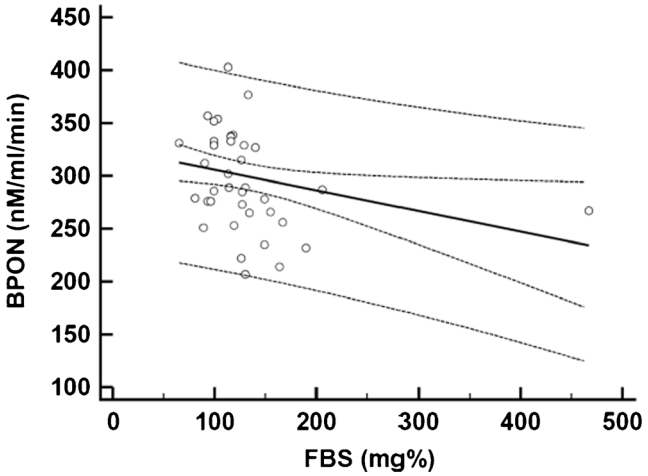

Fig 5 Showing the scatter diagram with regression line for FBS and BPON case group

SOD (r^2=− 0.40); P<0.05). In contrast, plasma MDA levels are positively correlated with both fasting and post-prandial blood glucose levels (r^2=0.50; P<0.01). Fasting blood sugar has significant negative correlation with BPON activity in cases (r^2=0.3; P<0.05). The correlation between HDL and MDA was not strong, but HDL: PON ratio is significant positively correlated with MDA in case group (r^2=0.48, P<0.01), whereas it was reverse in control group (r^2=−0.40, P<0.01) (Table 4).

Discussion

Hyperglycemia induced oxidative stress:

In diabetes [33] high levels of glucose have been associated with the production of ROS through its auto oxidation, advanced glycoprotein end products, increased

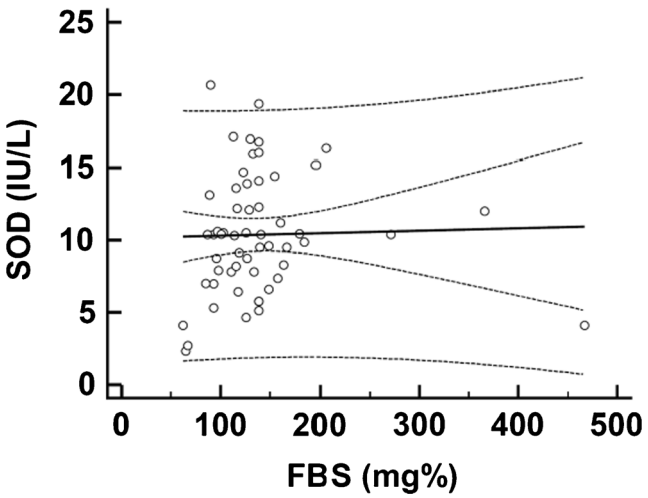

Fig 6 Showing the scatter diagram with regression line for FBS and SOD case group

Table 3 Showing the summary statistics of various parameters for both patients and controls

		Age (Y)	FBS (mg%)	PPBS (mg%)	% HbA_{1C}	TC (mg%)	TGL (mg%)	LDL (mg%)	HDL (mg%)	BPON (μM/mL/min)	HDL : PON	SOD (IU/mL)	MDA (μmol/L)
TYPE 2 DM (n=56)	Mean±2SD	58±10	139±66	229±84	7.99±1.26	167±36	153±89	106±32	37±7.5	284±59	0.14±0.05	10.38±4.17	111±35
	95 % CI	55–60	121–156	206–251	7.65–8.35	158–177	129–177	98–115	34–38	267–299	0.122–0.15	9.26–11.5	102–121
	P-value Normal Dist	0.53	0.003*	0.036*	0.02*	0.86	0.22	0.80	0.78	0.74	0.08	0.36	0.26
CONTROLS (n=29)	Mean±2SD	42±6	99±14	106±21	5.2±0.58	180±31	107±49	124±29	39±8	371±46	0.10±0.026	16.91±2.89	63±12
	95 % CI	40–45	93–104	98–114	4.96–5.4	167–191	88–125	113–135	36–42	353–388	0.096–0.116	15.8–18	59–68
	P-value Normal Dist	0.57	0.42	0.1	0.86	0.65	0.5	0.99	0.8	0.82	0.64	0.13	0.54
	t-test (p-Value)		< 0.01**	< 0.01**	< 0.01**	> 0.05	< 0.05*	> 0.05	> 0.01	< 0.0001***	< 0.05*	< 0.0001***	< 0.0001***

*statistically significant, ** statistically highly significant, *** statistically very highly significant

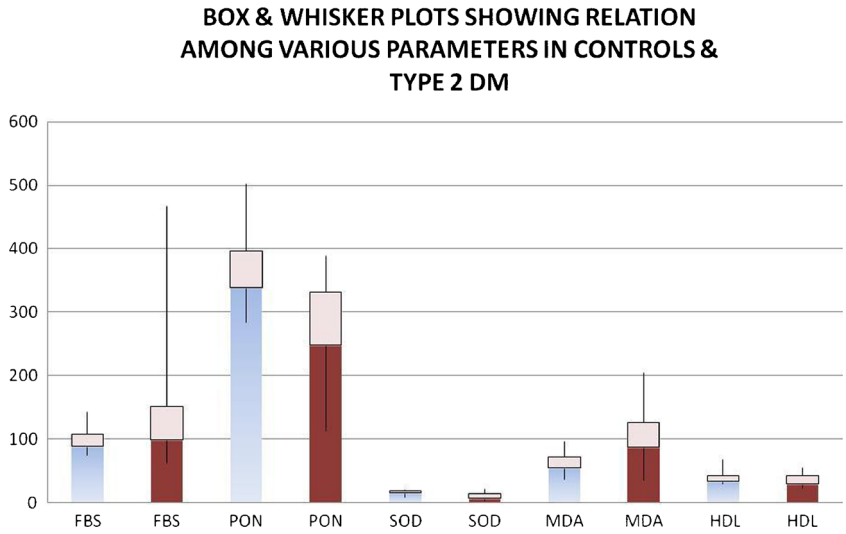

Fig. 7 Box and whisker plot. The boxes extend between the 25th and 75th percentile. The medians of the two groups are linked for comparison. The whiskers indicate the minimum and maximum value for the two groups

diversion of glucose to sorbitol (polyol pathway) and hexosamine stimulated secretion of several proinflammatory cytokines, particularly Tumor Necrosis Factor Alpha (TNFα) and several other interleukins [34]. ROS produced in diabetes is large, which cannot be neutralized by the antioxidant system [35]. In addition, the unprocessed ROS may cause irreversible damage to bio molecules such as phospholipids and leads to the initiation of lipid peroxidation, which in turn leads to CVD risk [36]. The ultimate stable end product of lipid peroxidation is malondialdehyde (O=HC-CH2-CH=O), a highly reactive carbonyl end product [37, 38]. Hence, MDA is a reliable biomarker for assessing oxidative stress. In our study all diabetic patients were under poor glycemic control (FBS—140 mg%, PPBS—284 mg%, HbA_{1C}—7.99 %). In this study we sought to correlate between hyperglycemia and lipid peroxidation end products. MDA levels are significantly higher (113±38 μM/L) in diabetes patients when compared with healthy controls (64±13 μM/L)). We have found a positive linear relationship between glucose levels and MDA levels (r^2—0.5, P<0.001). FBS proved to be a determining factor for the elevated MDA levels. Age did not have any effect on MDA levels (Table 2, Fig. 1).

Table 4 Showing the correlation coefficient (r^2) between different parameters

r^2	MDA		BPON		SOD	
	Case	Control	Case	Control	Case	Control
Age	0.07	0.05	− 0.19	0.16	− 0.08	− 0.5*
FBS	0.5**	0.1	− 0.3*	0.19	0.03	− 0.09
PON	− 0.53**	0.16	–	–	–	–
SOD	− 0.40*	0.01	0.34*	−0.16	–	–
HDL	− 0.23	− 0.03	0.2	0.06	− 0.1	− 0.08
HDL:PON	0.48**	− 0.40**	–	–	− 0.3*	–

*statistically significant ** statistically highly significant

MDA Vs HDL:

MDA reversibly binds to macromolecules such as amino acids of proteins, DNA and alters their function by forming malondialdehyde adducts. MDA may change the structure and functional property of apoA-I by forming lysine adducts [16]. This may affect the interaction of apo A1 with PON which might in turn affect PON activity. Aviram et al. proposed that, PON may lose its enzymatic functions due to oxidative stress [8]. In the present study, apoA-I associated enzyme, paraoxonase was decreased (284±59 nM/mL/min) among patients when compared with controls (371±46 nM/mL/min). In addition, PON activity is negatively correlated with MDA levels (r^2=− 0.53, P<0.001) but not with HDL concentration. Whereas HDL: PON ratio was increased in patients when compared with controls, suggests that, MDA has a profound effect on the composition of HDL and its function. Similar results were found in the study of Nair SP et al.

We propose that, the lipid peroxidation end products may play a role in the disintegration of HDL complex leading to a decrease in paraoxonase activity [39].

SOD activity during oxidative stress

SOD is a prime antioxidant enzyme to eliminate superoxide radicals produced in vivo. It scavenges superoxide radicals by catalyzing the conversion of two of these radicals into the less toxic hydrogen peroxide and molecular oxygen in the presence of Mn [40]. This enzyme levels were consumed in diabetes. It was surprising that,

the increased levels of H_2O_2 suppress the activity of SOD by irreversible oxidation of Cu metal cofactor associated with it [41]. A similar phenomenon was observed in our study , serum levels of SOD were significantly decreased (10.38±4.33 IU/mL) in patients when compared with control subjects (16.91±2.89 IU/mL) and those levels are negatively correlated with plasma MDA levels (r^2=−0.40; p<0.01). In contrast with these results, the activity of SOD among 15 very poorly controlled DM was fairly increased (12.8 IU/mL). The increase in SOD activity in uncontrolled diabetes is not yet clear. It has been proposed that, SOD can be rapidly induced in some conditions when cells exposed to ROS [42].

Association between HDL levels and BPON activity

The anti atherogenic property of HDL is due to the presence of PON, which is the principal protein among several antiatherogenic proteins associated with it. It protects both HDL and LDL from oxidative attack. Serum PON1 activity is reduced in diabetes, familial hypercholesterolaemia, and conditions associated with accelerated atherogenesis [23, 43]. Although serum PON activities and concentrations have been correlated with HDL-C and apoA-I levels, the relationship is not strong [23]. However, when serum HDL levels are only moderately decreased, the decrease in PON is independent of changes in HDL concentration [23]. Similar results were found in our study, serum levels of HDL were not significantly differed between the groups, but the levels were below the physiological range. The PON activity is decreased in patients when compared with controls, although there no significant difference in the levels of HDL. Watson et al. reported that during an acute phase reaction, there is a significant loss of the PON activity, accounting for production of dysfunctional HDL, which is incapable to protect LDL from oxidation [21]. More recently, Navab and colleagues reported that, HDL fail to protect LDL from oxidation in patients with coronary atherosclerosis, and they proposed that, it is due to their low serum PON1 activity despite relatively normal HDL levels [44]. In our study, PON activity parallels with SOD activity, again these both were negatively correlated with plasma MDA and glucose levels among cases. This confirms that, the decrease in HDL quality due to hyperglycemia induced oxidative stress and decreased serum antioxidant status.

The factors modifying the serum PON1 concentration are not yet clear. Diabetes mellitus is associated with low PON, in which the decrease in enzyme activity is more profound than the decrease in HDL concentration [43]. Serum PON1 activity is therefore likely to hinge on the number of PON1 molecules in HDL rather than the serum HDL concentration. The greatly increased ratio of apo AI to PON1 mass found in the MI patients would tend to support this argument [45, 46].

Conclusion

From our findings we conclude that, hyperglycemia induced oxidative stress could initiate the disintegration of anti-atherogenic molecules such as HDL in vivo. The altered HDL has a reduced capacity to protect other molecules such as LDL against oxidative modification. The most important finding in the present study is that in poorly controlled diabetics there is a significant fall in the anti-oxidant and anti-atherogenic enzyme associated with HDL namely paraoxonase. This fall is not related to a concomitant fall in the corresponding HDL levels. Large scale studies could be undertaken to further substantiate the mechanisms involved in the decline of serum paraoxonase.

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