

Antioxidant Enzymes and Lipid Peroxidation in Type 2 Diabetes Mellitus Patients with and without Nephropathy

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Abstract

Background: Oxidative stress has been considered to be a pathogenic factor of diabetic complications including nephropathy. There are many controversies and limited studies regarding the antioxidant enzymes in diabetic nephropathy. **Aim:** This study was to evaluate the levels of antioxidant enzymes and lipid peroxidation in Type-2 Diabetes Mellitus (DM) patients with and without nephropathy. **Materials and Methods:** The study included 90 age and sex matched subjects. Blood samples of all subjects were analyzed for all biochemical and oxidative stress parameters. **Results:** The malondialdehyde (MDA) levels and catalase (CAT) activity were significantly increased and reduced glutathione (GSH) levels and activities of glutathione peroxidase (GPx) and glutathione reductase (GR) were significantly decreased in Type-2 DM with and without nephropathy as compared to controls and also in Type-2 DM with nephropathy as compared to Type-2 DM without nephropathy. There were an excellent positive correlation of glycohemoglobin (HbA1c) with MDA and a good negative correlation of GPx with GSH in controls. There were positive correlations of GR, CAT, and superoxide dismutase (SOD) with MDA in Type-2 diabetes patients with nephropathy. **Conclusions:** Intensity of oxidative stress in Type-2 diabetic patients with nephropathy is greater when compared with Type-2 diabetic patients without nephropathy as compared to the controls.

Keywords: Antioxidant enzymes, Glycosylated hemoglobin and diabetic nephropathy, Lipid profile, Malondialdehyde, Non-insulin dependent diabetes mellitus, Reduced glutathione

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Introduction

Oxidative stress is an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free

radicals that damage all components of the cell, including proteins, lipids, and deoxyribonucleic acid (DNA). Further, some reactive oxidative species act as cellular messengers in redox signaling. Thus, oxidative stress can cause disruptions in normal mechanisms of cellular signaling. Chemically, oxidative stress is associated with increased production of oxidizing species or a significant decrease in the effectiveness of antioxidant defenses, such as glutathione.^[1,2] The effects of oxidative stress depend upon the size of these changes, with a cell being able to overcome small perturbations and regain its original state. However, more severe oxidative stress can cause cell death and even moderate oxidation can trigger apoptosis, while more intense stresses may cause necrosis.^[3] The major portion of long term effects is inflicted by damage on DNA.^[4] However, under the severe levels of oxidative stress that cause necrosis, the

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damage causes adenosine triphosphate (ATP) depletion, preventing controlled apoptotic death and causing the cell to simply fall apart.^[5,6]

Hyperglycemia can increase oxidative stress through several pathways. A major mechanism appears to be the hyperglycemia-induced intracellular reactive oxygen species (ROS), produced by the proton electromechanical gradient generated by the mitochondrial electron transport chain and resulting in increased production of superoxide.^[7] Two other mechanisms have been proposed that may explain how hyperglycemia causes increased ROS formation. One mechanism involves the transition metal-catalyzed autoxidation of free glucose, as described in cell-free systems. Through this mechanism, glucose itself initiates autoxidative reaction and free radical production yielding superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2).^[8] The other mechanism involves the transition metal-catalyzed autoxidation of protein-bound Amadori products, which yields superoxide and hydroxyl radicals and highly reactive dicarbonyl compounds.^[9]

Oxidative stress is hypothesized to play a role in the development of diabetes with and without nephropathy. Oxidative stress has been considered to be a pathogenic factor of diabetic complications including nephropathy. The intensification of free-radical reactions in patients with type 2 diabetes was a factor responsible for the development of vascular changes.^[10-12]

The development of vascular complications in diabetes correlates with the intensity of hyperglycemia. High intracellular glucose concentration has been suggested to be a prerequisite for the development of functional and structural changes in the kidney typical of diabetic nephropathy. Under the conditions of intracellular hyperglycemia, the cellular NADH/NAD⁺ ratio is decreased (pseudohypoxia). Activation of free radical production is one of the numerous biochemical sequelae of pseudohypoxia.^[13] Oxygen-derived free radicals have been suggested to induce malfunction of endothelial cells leading to diabetic angiopathy.^[14]

Although there is better understanding of the biochemical and molecular mechanisms responsible for the development of diabetic nephropathy, its multifactorial pathogenesis still raises numerous questions. The aim of this study was to investigate the process of lipid peroxidation and activities of key antioxidant enzymes in patients with Type 2 diabetes mellitus (DM) with or without nephropathy.

Materials and Methods

The case control study included 90 subjects with age and sex matched (having 30 healthy control subjects (group I),

30 Type 2 DM patients with (group III), and 30 Type 2 DM patients without nephropathy (group II)). The study was approved by the institutional ethical committee and written consent was also taken from the patients.

Thirty age and gender matched diabetics (without nephropathy) with normal albumin excretion rate (<20 $\mu\text{g}/\text{min}$) was taken. These Type 2 DM patients attending the out patient department (OPD) and indoor of Department of Medicine, GR Medical College, Gwalior were diagnosed on the basis of history, physical examination, and biochemical investigations and according to the biochemical criteria laid down by the revised American Diagnostic Association Criteria (ADA)^[15] and dyslipidemia by using National Cholesterol Education Program (NCEP).^[16]

Thirty Type 2 DM patients with nephropathy with age and gender-matched with persistent proteinuria, over 0.5 g/day and urinary albumin excretion rate over 200 $\mu\text{g}/\text{minute}$ were diagnosed on the basis of history, physical examination and biochemical investigations done by the physician. In addition to insulin and hypoglycemic drugs, all patients with obvious proteinuria (Type 2 DM with nephropathy) received anti-hypertensive medication such as calcium antagonists, angiotensin converting enzyme inhibitors, and vasodilators. Thirty age and gender matched healthy individuals considered as controls.

Patients with acute and chronic inflammatory conditions, cerebrovascular accident as well as smoking, obesity (body mass index (BMI) >30 kg/m^2) and alcoholics were excluded from the study. None of the subjects were on antioxidant supplementation or lipid lowering drugs.

Height and weight were noted for the calculation of BMI [(BMI = weight in kilograms/height in meters)²].

Under all aseptic conditions, venous blood sample was collected from each subject, by disposable syringe. The samples were stored at + 2°C to + 8°C (+4°C) before analysis and all the samples were analyzed on the same day of collection. This sample was distributed in following vials:

Heparinized sample for estimation of reduced glutathione (GSH), citrated sample for estimation of plasma malondialdehyde (MDA), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx), serum for estimation of superoxide dismutase (SOD), lipid profile, Ethylene di-amine tetra acetic acid (EDTA) sample for estimation of glycosylated hemoglobin (HbA1c) and fluoride samples for Fasting

plasma glucose (FPG), and post prandial plasma glucose (PPPG).

Glycosylated hemoglobin was measured by latex agglutination inhibition assay.^[17,18] Fasting and post prandial plasma glucose was estimated by method of Glucose Oxidase-Peroxidase (GOD-POD) by Trinder.^[19] The serum urea and creatinine were measured by routine spectrophotometric methods.

Reduced glutathione

GSH level was estimated by the method of Beutler *et al.*,^[20] using 5-5'-dithiobis-(2-nitrobenzoic acid) (DTNB).

Glutathione peroxidase

GPx activity was measured by using hydrogen peroxide as a substrate by applying the method of Hafeman *et al.*^[21]

Glutathione reductase

GR catalyses the reduction of Glutathione disulfide (GSSG) in the presence of NADPH, which is oxidized to NADP⁺. GR level was estimated by the method of Horn HD.^[22]

Catalase

CAT was estimated by the method of Sinha.^[23] The method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of hydrogen peroxide (H₂O₂), with the formation of perchromic acid as an unstable intermediate. The chromic acetate thus produced is measured colorimetrically.

Superoxide dismutase

The activity of SOD was assayed by applying the method of Mishra *et al.*^[24] The ability of superoxide dismutase to inhibit the auto-oxidation of epinephrine at pH 10.2 has been used as the basis of a convenient and sensitive assay for this enzyme.

Malondialdehyde

Plasma MDA was estimated by method of Jean *et al.*^[25] After the reaction of thiobarbituric acid with malondialdehyde, the reaction product was extracted in butanol and was measured.

Statistical analysis

All results were expressed in Mean \pm SD. Differences between the mean were calculated by analysis of variance (ANOVA) test (multiple comparisons). The statistical analysis was performed using Statistical Product and Service Solutions (SPSS) version 17.0. Pearson correlation coefficients were calculated to analyze relationships between biochemical

parameters. The level of significance was considered as *P* value < 0.05.

Results

The levels of Triglyceride (TG), Very low density lipoprotein cholesterol (VLDL-C), and MDA (*P* < 0.001) as well as BMI (*P* < 0.05) were significantly increased and High density lipoprotein cholesterol (HDL-C) and GSH (*P* < 0.001) were significantly decreased in Type 2 Diabetic Patients with and without Nephropathy as compared to controls.

The levels of TG (*P* = 0.02), VLDL-C (*P* = 0.034), and MDA (*P* = 0.035) were significantly increased and HDL-C and GSH (*P* < 0.001) were significantly decreased in Type 2 Diabetic Patients with Nephropathy as compared to Type 2 Diabetic Patients without Nephropathy.

The levels of HbA1c *P* < 0.001, FPG and CAT (*P* = 0.006), PPPG (*P* = 0.01), Urea, Creatinine (*P* < 0.05) were significantly increased and activities of GPx, GR (*P* < 0.001) were significantly decreased in Type 2 DM without nephropathy as compared to controls.

The levels of HbA1c, FPG, PPPG, Urea, Creatinine, TC, Low density lipoprotein cholesterol (LDL-C), and activity of CAT were significantly increased (*P* < 0.001) and activities of GPx, GR, and SOD were significantly decreased (*P* < 0.001) in Type 2 DM with nephropathy as compared to controls and type 2 DM without nephropathy.

There were no significant changes in the levels of TC (*P* = 0.80), LDL-C (*P* = 0.82) and activity of SOD (*P* = 0.87) and activities of GPx and GR were significantly decreased, whereas CAT was significantly increased in Type 2 DM without nephropathy as compared to controls [Table 1].

There were an excellent positive correlation of HbA1c with MDA (*r* = 1.000, *P* = 0.0001) and a good negative correlation of GPx with GSH (*r* = -0.625, *P* = 0.0001) in controls. There were fair positive correlation of GR, good positive correlation of CAT and SOD with MDA (*r* = 0.37, *P* = 0.04; *r* = 0.68, *P* = 0.001; *r* = 0.67, *P* = 0.001, respectively) in type 2 diabetic patients without nephropathy. There were fair positive correlations of FPG and PPPG with MDA (*r* = 0.49, *P* = 0.006; *r* = 0.37, *P* = 0.04, respectively), and fair negative correlation of TG and fair positive correlation of SOD with GSH (*r* = -0.39, *P* = 0.03; *r* = 0.41, *P* = 0.02, respectively) in type 2 diabetes patients with nephropathy [Table 2].

Table 1: Biochemical and oxidative stress indices of controls and type 2 diabetic patients with and without nephropathy

Variables	Controls (Group I, n=30)	Type 2 diabetic patients (Group II, n=30)	Type 2 diabetic patients with nephropathy (Group III, n=30)	P value
Age (years)	53.38±10.23	51.78±11.97	52.99±10.79	>0.05 ¹²³
Gender (M/F)	14/16	12/18	13/17	>0.05 ¹²³
Body mass index (kg/m ²)	23.0±0.9	27±1.0	26.1±0.9	<0.05 ¹² >0.05 ³
HbA1c (%)	4.69±0.72	6.86±0.61	7.58±0.44	<0.001 ¹²³
FPG (mmol/L)	5.09±0.41	6.02±0.63	9.07±2.08	0.006 ¹ <0.001 ²³
PPPG (mmol/L)	6.64±0.6	7.96±0.65	14.87±3.59	0.01 ¹ <0.001 ²³
Urea (mmol/L)	8.27±2.10	12.17±2.83	22.51±4.92	<0.05 ¹²³
Serum creatinine (µmol/l)	76±4.20	93±11.23	127±28.49	<0.05 ¹²³
TC (mmol/L)	4.95±0.57	5.03±0.62	6.98±1.92	0.80 ¹ <0.001 ²³
TG (mmol/L)	1.27±0.15	2.10±1.01	2.55±0.91	<0.001 ¹² 0.02 ³
HDL-C (mmol/L)	1.25±0.25	0.999±0.217	0.995±0.211	<0.001 ¹² 0.95 ³
LDL-C (mmol/L)	3.11±0.57	3.04±0.58	4.80±1.84	0.82 ¹ <0.001 ²³
VLDL-C (mmol/L)	0.58±0.07	0.98±0.45	1.18±0.42	<0.001 ¹² 0.034 ³
GSH (mg %)	14.21±2.55	12.20±1.84	10.62±1.84	<0.001 ¹² 0.005 ³
GPx (U/gm Hb)	8.44±1.17	5.92±0.64	4.08±1.60	<0.001 ¹²³
GR (U/gm P)	16.53±0.41	15.24±0.73	14.34±1.06	<0.001 ¹²³
CAT (U/gm P/mL)	5.79±0.58	6.68±0.97	7.81±1.75	0.006 ¹ <0.001 ²³
SOD (U/gm P/mL)	6.67±1.22	5.72±0.98	4.18±1.2	0.87 ¹ <0.001 ²³
MDA (nmol/mL)	4.69±0.72	7.09±1.15	7.87±2.03	<0.001 ¹² 0.035 ³

The values are mean±Standard deviation (SD); P Values: ¹p, Control vs. Type 2 Diabetic Patients; ²p, Controls vs. Type 2 Diabetic Patients with Nephropathy; ³p, Type 2 Diabetic Patients vs. Type 2 Diabetic Patients with Nephropathy; HDL-C: High density lipoprotein cholesterol; GSH: Glutathione

Discussion

Many studies have shown that increased oxidative stress is present in diabetic subjects.^[2,26-28] Consistent with this view, our data provides further evidence that there is presence of oxidative stress with an alteration in antioxidant enzyme activities and increased lipid peroxidation (MDA levels) in Type 2 diabetic patients. Diabetic nephropathy (DN) is one of the most important microvascular complications of diabetes and a major cause of end stage renal disease.^[29] Many pathways have been involved in pathogenesis of DN including oxidative stress,^[30] activation of protein kinase C,^[31] increased production of advanced glycation end products (AGE),^[32] and polyol-hexosamine pathway flux.^[33] The extreme production of reactive oxygen species (ROS) has been suggested as a common result leading to intensified oxidative damage at the level of lipid peroxidation^[34] and peak in DN in association with diabetes.^[35] Thus, any treatment that can stabilize oxygen metabolism and regulate oxidative stress can attenuate and delay the development of DN.^[36]

Hyperglycemia in diabetic patients can increase production of free radicals through Amadori rearrangement.^[37] In general, the ROS are continuously generated in physiological conditions and are eliminated by several antioxidant enzymes. Increasing oxidative stress can be an important contributing factor in the

pathogenesis of periodontal disease and diabetes. Therefore, the co-existence of both conditions could pathologically increase the effect of oxidative stress.^[38,39] In DN, excessive free radical generation has been shown to decrease the activities of antioxidant enzymes. The decrease in the GSH and GPx level was correlated with GR activity in DN. GPx catalyzes the reduction of lipid peroxides at the expense of GSH. H₂O₂ is also being inactivated by GPx resulting in an increased consumption of GSH.^[2] Increased activity of aldose reductase pathway in DN consumes NADPH, resulting in impaired activity of GR. Thus depletion of GSH impairs the activity of antioxidant enzymes as well as that of chain breaking aqueous and lipid phase antioxidants. The resultant oxidative damage can then contribute to pathogenesis of DN.^[40] Some authors have reported a decrease in SOD level in blood.^[40-42] We have also quoted decrease in serum SOD levels in our study. The reduction in serum SOD activity levels could be due to excessive consumption in the autoxidation procedure and increased excretion from the inflammatory kidney in nephropathy. Significant reduction in SOD activity could be linked to progressive glycation of enzymatic proteins. About 50% of SOD in erythrocytes of diabetic patients is glycated, resulting in low activity of SOD.^[43] The decrease in SOD activity may lead to increase level of superoxide radicals which will cause the inactivation of GPx.^[41] Selvam, *et al.*, reported the increase in CAT activity, which is to overcome the damaging effect of

Table 2: Correlation between the biochemical parameters with GSH and MDA in Controls, type 2 diabetic patients with and without nephropathy

Controls (Group I, n=30)		
Parameters	GSH (mg %)	MDA (n mol/mL)
HbA1C (%)	-	r=1.000**, P=0.0001
GPx (U/gm Hb)	r=-0.625**, P=0.0001	-
Type 2 Diabetic patients (Group II, n=30)		
GR (U/gm P)	-	r=0.37*, P=0.04
CAT (U/gm P/mL)	-	r=0.68**, P=0.001
SOD (U/gm P/mL)	-	r=0.67**, P=0.001
Type 2 Diabetic patients with Nephropathy (Group III, n=30)		
FPG (mmol/L)	-	r=0.49**, P=0.006
PPPG (mmol/L)	-	r=0.37*, P=0.04
TG (mmol/L)	r=-0.39*, P=0.03	-
SOD (U/gm P/mL)	r=0.41*, P=0.02	-

*Pearson correlation is significant at the 0.05 level (2 - tailed); **Pearson Correlation is significant at the 0.01 level (2-tailed); HbA1c: Glycosylated Hemoglobin; FPG: Fasting plasma glucose; PPPG: Postprandial plasma glucose; TC: Total cholesterol; TG: Triglyceride; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol; VLDL-C: Very low density lipoprotein cholesterol; CAT: Catalase; MDA: Malondialdehyde; GSH: Reduced glutathione; GR: Glutathione reductase; GPx: Glutathione peroxidase; SOD: Superoxide dismutase

erythrocyte membrane from oxidative attack.^[44] There is compensatory increase in CAT activity besides GPx in cell. CAT disposes off the H₂O₂ formed in the cell so that fragility of red blood cells (RBC) is maintained. The increased CAT activity in diabetes is an indication of the increased production of peroxide radicals.^[45]

In the present study, we have observed the increased thiobarbituric acid reacting substances (TBARS) in DN, the hyperglycemia associated with hyperlipidemia could be the causative factor for the increased production of free radicals and lipid peroxides i.e., MDA.^[46] In patients with DN, there is an increased production of free radicals which promotes lipid peroxidation. The intermolecular cross linking of collagen through MDA leads to its stabilization and allows further glycation. This in turn increases the potential of glycated collagen to initiate further lipid peroxidation.^[47] Also, defective pentose phosphate shunt in uremic red blood cells led to the peroxidation of polyunsaturated fatty acids in the cell membrane.

HbA1c can provide an accurate and reliable method to routinely assess the relative level of diabetes control. This finding was also supported by an evidenced based study

which showed a graded relationship between HbA1c and the risk of nephropathy in type 2 DM patients. Lipoprotein abnormalities are more pronounced in patients with high HbA1c levels.^[48]

DN is associated with an altered lipid profile characterized by elevated triglyceride rich lipoproteins even in the early stages of renal disease. In this study, the plasma total cholesterol, TG, LDL-Cholesterol, VLDL-Cholesterol were significantly higher in DN than in diabetics without nephropathy while in HDL-Cholesterol, there was no significant changes in Diabetic nephropathy than in diabetics without nephropathy. Bonnet *et al.* also reported similar finding in both types of diabetes indicating an independent deleterious influence of serum total cholesterol on the decline in renal function and progression of albuminuria.^[49] According to Martinez-Castelao *et al.*, (2002) low levels of HDL-Cholesterol is a key feature of Type 2 diabetes.^[50]

Conclusion

The decreased efficiency of cellular antioxidant mechanisms in Type 2 DM patients with nephropathy with simultaneous enhanced lipid peroxidation may constitute the pathogenic link between hyperglycemia and development of endothelial dysfunction. Moreover, the extent of oxidative stress and insufficiency of defensive antioxidant mechanisms in Type 2 Diabetic patients is dependent on the metabolic control of diabetes and the occurrence of complications.

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