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Original Article

Alterations in some Oxidative Stress Markers in Diabetic Nephropathy

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ABSTRACT

Background: Diabetic nephropathy is the most common cause of end stage renal disease. Oxidative stress is being considered as a common pathogenic factor in diabetes mellitus and its complications. **Aim:** To assess the level of oxidative and antioxidative markers in Type 2 diabetes mellitus patients with micro albuminuria and without micro albuminuria, in North West Indian ethnic population. **Material and Method:** Serum level of malondialdehyde (MDA), superoxide dismutase (SOD), reduced glutathione (GSH), glutathione peroxidise (GPx) & glutathione reductase (GR) were estimated in controls (Group-1), Type 2 diabetes patients without micro albuminuria (Group-2) and Type 2 diabetes patients with micro albuminuria (Group-3). **Results:** Serum MDA level was significantly increased by 204.71 ($p < 0.001$) and 291.09% ($p < 0.001$) in Type 2 diabetes patients without (Group-2) and with micro albuminuria (Group-3) with respect to control subjects (Group-1). A significant increase in MDA levels by 28.35% ($p < 0.05$) was found in Group-3 in comparison to Group-2. The activity of SOD, GSH, GR and GPx was significantly reduced by 46.01% ($p < 0.01$) in Type 2 diabetes patients without and with micro albuminuria in comparison to healthy control group. A similar trend of significant decrease in SOD, GSH, GR and GPx levels was also recorded in Group-3 with respect to Group-2. **Conclusion:** The results of present study suggested that oxidative stress increases in diabetic patients. Further micro albuminuria accelerates the oxidative stress in these patients and hence could be responsible for the phathophysiology of various vascular complications. Larger studies need to be undertaken to substantiate the above mentioned findings.

Key Words: Micro albuminuria, Malondialdehyde (MDA), Superoxide Dismutase (SOD), Glutathione (GSH), Diabetic nephropathy.

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INTRODUCTION

World Health Organization reported that diabetes affects more than 170 million people worldwide and this number will rise to 370 million by 2030.[1] Microvascular and macrovascular complications of diabetes mellitus are the leading cause of mortality and morbidity.[2,3] Diabetic nephropathy is the most common cause of end stage renal disease.[4] Many biological pathways, such as glucose autoxidation, polyol pathway, prostanoid synthesis and protein glycation are triggered in hyperglycaemia state leading to increased production of free radicals.[5] Oxidative stress is being considered as a common pathogenic factor in diabetes mellitus and its complications.[6,7,8,9] The most important free radicals that cause oxidative stress are superoxide, hydroxyl radical and hydrogen peroxide. In human, there are antioxidant enzymes like superoxide dismutase (SOD), catalase, and glutathione (GSH) and its enzymes such as glutathione reductase (GR), glutathione peroxidise (GPx) & glutathione-s-transferase, which scavenges the action of free radicals in order to protect the body. When the generation of reactive oxygen species (ROS) exceeds cellular defence mechanisms, these unstable molecules interact with biologic macromolecules such as lipids, proteins and DNA and lead to structural changes as well as functional abnormalities. A number of studies have estimated the status of oxidative stress in diabetics. Some researchers have reported oxidative damage in Diabetic mellitus patients.[10,11,12,13,14] On the other hand some researchers have reported no significant change in oxidative stress in diabetic patients.[15,16] Moreover data in patients of North West Indian ethnic origin, on oxidative stress in Type 2 diabetes mellitus patients with early nephropathy is scant. So, the present study was undertaken to assess the level of oxidative (malondialdehyde) and anti-oxidative (SOD, GSH, GR & GPx) markers in Type 2 diabetes mellitus patients with micro albuminuria and without micro albuminuria.

MATERIAL AND METHOD

The study was conducted in the department of Biochemistry, Govt. Medical College, Amritsar (Punjab) - India on Type-2 diabetes mellitus patients with and without micro albuminuria, of both sexes. Patients within the age group 35 - 55 years and minimum of one year duration of diabetes attending O.P.D. and wards of the Medicine Department in Guru Nanak Dev Hospital, Amritsar were inducted in the study. Diagnosis of diabetes was made according to revised American Diabetic Association criteria. Fifty age and sex matched normal healthy subjects with no family history of diabetes were selected from the general population as control group. Subjects were divided into following three groups:

Group -1(n=50): Normal healthy subjects (Control group)

Group -2 (n=50): Type-2 Diabetes Mellitus patients without micro albuminuria

Group-3 (n=50): Type-2 Diabetes Mellitus patients with micro albuminuria

Type-2 diabetes mellitus patients with albumin/creatinine ratio 30 to 300 mg/g were considered as having micro albuminuria. Positive results on two of three tests (30 to 300 mg of albumin per g of creatinine) in a six-month period were considered diagnostic for diabetic nephropathy.)

Exclusion Criteria

Diabetic patients with blood pressure >130/80mm Hg and other causes of proteinuria like fever, urinary tract infection, prostatitis, congestive heart failure and hematuria etc., those on Angiotensin Receptor Blocker

& ACE inhibitors and patients on Vitamin A, C and E supplements abd cases of gestational diabetes mellitus were excluded from the study.

Blood sampling

Venous blood samples (10 ml) were drawn from all the subjects following an overnight fast of 12 h under all aseptic conditions in sterile, dry and acid washed vial. The collected blood sample was divided in three sets of vials to assess the different biochemical assays as described below.

2 ml of blood was collected into potassium oxalate and sodium fluoride mixture (1:3 ratio) containing vial and then centrifuged at 3000 rpm for 15 min at 4 °C. The supernatant (plasma) obtained was used for the analysis of fasting glucose levels.

4 m1 of the blood sample was collected into heparinized vials and centrifuged at 3000 rpm for 15 min at 4 °C and the supernatant (plasma) obtained was used for the analysis of malondialdehyde and superoxide dismutase levels and heparinized whole blood sample was used for the estimation of HbA_1c, reduced glutathione, glutathione peroxidase and glutathione reductase.

4 ml of the blood was collected in acid washed sterile vials, this sample was allowed to clot & then centrifuged at 3,000 rpm for 15 min at 4 °C and the serum obtained was used for the analysis of MDA levels.

Biochemical assays

Glucose

Glucose was estimated spectrophotometrically using an enzymatic test kit base by the method based on GOD-POD method supplied by Transasia Bio-Medicals Ltd. Solan (HP); in Technical collaboration with ERBA diagnostic Mannheim, Germany.

Glycosylated Hemoglobin (HbA_1C)

HbA_1C was analyzed by using kit supplied by Transasia Bio-Medicals Ltd. Solan (HP); in Technical collaboration with ERBA agnostic Mannheim, Germany based onion-exchange resin method in which a hernolyzed preparation of the whole blood is mixed continuously for 5 ruin with a weak binding cation exchanges resin. During this time, non-HbAlc binds to the resin. After the mixing period a filter is used to separate the supernatant containing the glvcohemoglobin from the resin. The glycohemoglobin percent is determined by measuring the absorption at 415 nm of the glycohemoglobin fraction and the total hemoglobin fraction. The ratio of the two absorbances gives the percentage glycohemoglobin.

Albumin

Albumin in urine was measured using standardized albumin kit supplied by Transasia Bio-Medicals Ltd. Solan (HP); in Technical collaboration with ERBA diagnostic Mannheim, Germany.

Creatinine

Creatinine in urine was measured using standardized creatinine kit supplied by Transasia Bio-Medicals Ltd. Solan (HP); in Technical collaboration with ERBA diagnostic Mannheim, Germany.

Urine Albumin/Creatinine ratio (ACR)

ACR was calculated by standard formula and value in the range of 30 – 300 mg/g was considered as micro albuminuria.

Anylaysis of Oxidative Stress Markers

Malondialdehyde (MDA)

MDA level in serum was assessed by the new colorimetric method of Satoh.[17] After the reaction of thiobarbituric acid with malondialdehyde, the reaction product was extracted in butanol and was measured.

Superoxide Dismutase (SOD)

SOD levels in plasma were estimated by the method of Marklund and Marklund, 1974[18] modified by Nandi and Chatterjee, 1988.[19] 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 the SOD enzyme.

Reduced Glutathione (GSH)

GSH levels in blood were assessed using 5-5'-Dithiobis (2-Dinitrobenzoic Acid (DTNB) by the method of Beutler *et al,* 196.[20]

Glutathione Peroxidase (GPx)

GPx activity in whole blood was estimated by the method of Paglia and Valentine using H_2O_2 as a substrate.[21]

Table 1: Biochemical and demographic profile of Type-2 Diabetes Mellitus patients with and without micro albuminuria and normal healthy control subjects

Anthropometric parameters	Group – 1 [Normal healthy subjects]	Group – 2 [Type-2 Diabetes Mellitus patients without micro albuminuria]	Group – 3 [Type-2 Diabetes Mellitus patients with micro albuminuria]
Male	28	27	29
Female	22	23	21
Body Mass Index (kg/m2)	27.89 ± 1.21	27.23 ± 1.43 (2.36)	29.95 ± 0.92 (7.38)b (9.98)c
Height (cm)	146.20 ± 1.45 a	151.34 ± 2.15a (+3.51)	149.5 ± 1.54a (+2.26)b (-1.20)c
Age (in years)	54.55 ± 5.87a	55.26 ± 4.18a (+1.30)b	54.91 ± 6.14 a (+0.66) b (-0.63)c
Weight (Kg)	67.23 ± 7.53a	71.28 ± 5.91a (+6.02) b	65.39 ± 8.29 a (-2.73)b (-7.78)c
SBP (mmHg)	120.11 ± 3.21a	122.92 ± 5.15 a (+2.34) b	121.44 ± 4.92 a (+1.11) b (-1.20)c
DSBP (mmHg)	85.23 ± 5.33a	84.31 ± 3.41 (-1.08) b	87.19 ± 5.83 a (+2.30) b (+3.42)c
Hemoglobin(g/dL)	15.04 ± 1.02a	14.31 ± 1.11 a (-4.83) b	14.96 ± 0.94 a (-0.53) b (+4.54)c

a: Values expressed as Mean ± SD of 50 observations.

b: Values in parentheses representing the percentage change w.r.t normal healthy control subjects (Group-1).

c: Values in parentheses representing the percentage change w.r.t Type-2 Diabetes Mellitus patients without micro albuminuria (Group-2).

Glutathione Reductase (GR)

GR activity was estimated by applying the method of Glodberg and Spooner.[22]

Statistical analysis

Results of biochemical analyses were using SPSS-17 program and expressed as mean value ± standard deviation (S.D.) continuous variables with normal distribution. The Student's "t" – test was used to test the significance of difference between the mean values of diabetic patients with & without micro albuminuria and normal healthy subjects. The differences were considered significant at $p<0.05$.

RESULTS

Biochemical and demographic profile of the patients and controls is summarized in Table 1. All the subjects in our study were matched for age, sex and Body Mass Index (BMI). Duration of diabetes and fasting blood sugar level and glycated haemoglobin levels were similar in both the patient groups.

Serum MDA level was significantly higher in patients of Type 2 diabetes both with micro albuminuria and without micro albuminuria with respect to control subjects. A significant increase in MDA levels by 28.35% ($p < 0.05$) was found in type - 2 diabetes mellitus patients with micro albuminuria in comparison to type-2 diabetes mellitus patients without micro albuminuria (Figure 1).

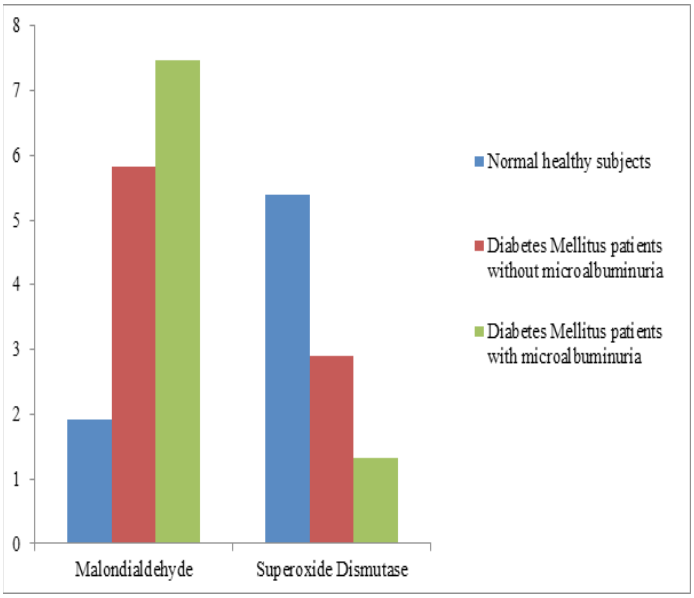

Figure 1: Alterations in malondialdehyde and superoxide dismutase levels in normal healthy subjects and type-2 diabetes mellitus patients with & without out micro albuminuria.

Values in figure are expressed as Mean ± Standard deviation. The levels of malondialdehyde and superoxide dismutase are expressed in nmol/ml and U/ml respectively.

The activity of SOD was significantly reduced by 46.01% ($p < 0.01$) in group 2 and 75.32% ($p > 0.001$) in group-3 in comparison to healthy control group (Group 1). A significant ($p < 0.01$) decrease by 54.29% was also observed in SOD levels in group-3 with respect to group 2 (Figure 1). The results of GSH, GR and GPx are summarized in figure 2. A significant decrease by 30.25% ($p < 0.01$) & 52.14% ($p < 0.001$) was found in GSH levels, by 32.02% ($p < 0.01$) & 57.73% ($p < 0.001$) in GR levels and by 41.85% ($p < 0.001$) & 60.52% ($p < 0.001$) in group-2 and group-3 respectively with respect to group 1. A significant fall in GSH, GR and GPx levels by 31.39%, 37.82% and 32.12% respectively was also observed in

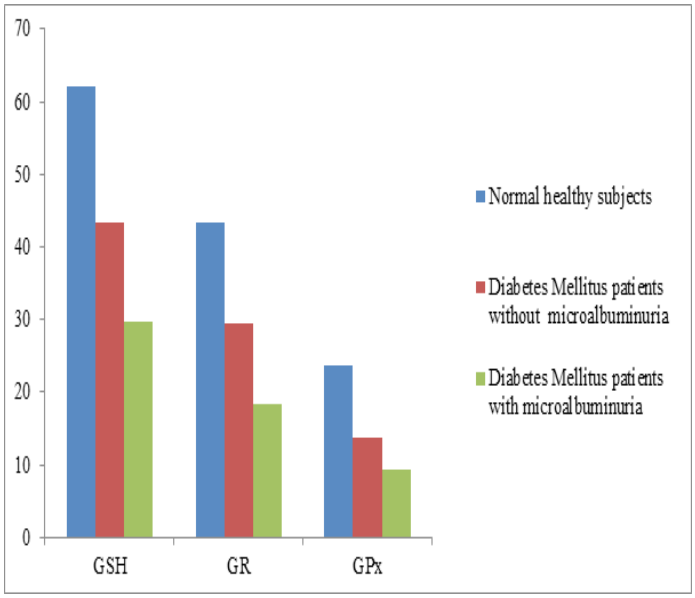

Figure 2: Alterations in reduced glutathione, glutathione reductase and glutathione peroxidase levels in normal healthy subjects and type-2 diabetes mellitus patients with & without out micro albuminuria.

Values in figure are expressed as Mean ± Standard deviation. The levels of reduced 3glutathione are expressed in mg/g Hb and levels of glutathione reductase and glutathione peroxidase are expressed in mU/g Hb.

Type 2 diabetic patients with micro albuminuria (Group 3) with respect to Type 2 diabetic patients without micro albuminuria (Group 2).

DISCUSSION

Recently free radical reactions are being considered as the unifying link between diabetes and its complications. In our study, we found significantly higher serum MDA levels in Type 2 diabetic patients. Serum MDA level was significantly higher in patients of Type 2 diabetes with micro albuminuria as compared to patients without micro albuminuria. This finding is in agreement to previous studies.[10,13,16,23] This significant increase in MDA levels could be due to increased production of free radicals in diabetes mellitus patients. MDA is an end product of lipid peroxidation could be responsible for the intermolecular cross linking of collagen through MDA leads to its stabilization and further glycation. This starts a vicious cycle as glycated collagen initiates further lipid peroxidation releasing more MDA.[24]

SOD, a superoxide radical scavenging enzyme, is considered the first line of defence against the deleterious effect of oxygen radicals in the cells. The presence of SOD in various compartments of the body enables it to dismutate O_2^- radicals immediately and protects the cells from oxidative damage. Serum SOD level was significantly decreased in patients of Type 2 diabetes with micro albuminuria as compared to patients without micro albuminuria. Literature supports our findings.[11,13,25] A significant inhibition in SOD activity in type-2 diabetic patients with micro albuminuria may results in an increased flux of O_2^- radical and hence reflects the tissue damage/injury.

Glutathione acts as a direct scavenger as well as co-substrate for GSH peroxidise. It is a major intracellular redox tampon system. In our study we observed that GSH levels were significantly lower in Type 2 diabetic patients as compared to controls. GSH levels were significantly lower in Group 3 subjects as compared to Group 2 subjects. Thus it indicates that depletion of GSH impairs the activity of antioxidant enzymes resulting in oxidative damage induced diabetic nephropathy.

Glutathione peroxidise, a selenium containing enzyme, catalyses the reduction of hydrogen peroxide using glutathione as substrate, thereby pre-

venting cells against oxidative stress. We observed significant decrease in GPx activity in diabetic patients as compared to controls. The decrease in level was more significant in patients with micro albuminuria. This observation is in accordance with the hypothesis that increase MDA level and decreased GPx level might play a role in tissue damage.[26,27,28]

CONCLUSION

The results of our present study suggested that oxidative stress increases in diabetic patients. Further micro albuminuria accelerates the oxidative stress in type -2 diabetic patients and hence could be responsible for the phathophysiology of various vascular complications. Larger studies need to be undertaken to substantiate the above mentioned findings.

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CONFLICT OF INTEREST

None

ABBREVIATIONS USED

DM: Diabetes Mellitus; MDA: Malondialdehyde; LPO: Lipid Peroxidation; SOD: Superoxide Dismutase; GSH: Reduced Glutathione; GR: Glutathione Reducatase GPx: Glutathione Peroxdase; H_2O: Water; $O_2^{\cdot-}$: Superoxide Anion; H_2O_2: Hydrogen Peroxide.

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