Research Article

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ANTIOXIDANT ENZYME LEVELS AS MARKERS FOR TYPE 2 DIABETES MELLITUS

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Abstract: Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterized by increased production of free radicals and oxidative stress. Oxidative stress can be defined as an imbalance between the removal of free radical production and altered activity levels of antioxidant enzymes. The aim of this study was to evaluate the antioxidant enzyme activity such as superoxide dismutase (SOD), catalase (CAT) and glutathione S transferase (GST) in T2DM patients and healthy subjects in a North Indian population. The study was conducted on 474 T2DM and 301 healthy subjects. Blood lysates were prepared and levels of antioxidant enzymes *viz.* SOD, CAT and GST were measured by spectrophotometric method. Data were analyzed by using Prism software (v 5.01) and expressed as Mean ± SE. The activity levels of SOD and GST were significantly lower in T2DM patients than in healthy subjects ($P<0.05$) whereas that of catalase was higher in T2DM patients (15.56±1.53 units/mg) than in healthy subjects (12.12±1.212 units/mg). Analysis of data also showed that enzyme activity levels decreased with increasing age both in normal and T2DM conditions. Only GST level showed significant decrease in diabetic males while both GST and SOD decreased in diabetic females. Assay of enzyme activity levels in erythrocytes could be used as markers to identify individuals predisposed to T2DM.

Keywords: Type 2 Diabetes mellitus, Antioxidant enzymes, Oxidative stress, Catalase, Superoxide dismutase, Glutathione S transferase

INTRODUCTION

Diabetes mellitus is a common metabolic disorder resulting from defects in insulin secretion, action or both. It is characterized by hyperglycemia often accompanied by glycosuria, polydipsia and polyuria [1-3]. According to Diabetes Atlas, 2011, 366 million people are affected with diabetes worldwide, and the number is likely to reach 552 million by the year 2030 with the largest increase in regions dominated by developing economies. The current reports revealed that India has 61.3 million diabetics which are expected to rise to 101.2 million by the end of 2030 [4, 5]. Type 2 diabetes mellitus (T2DM) is a heterogeneous metabolic condition that is attributed to an array of pathophysiological mechanisms, although insulin resistance (IR) and *β*-cell failure play key roles [6].

Free radicals and reactive oxygen species (ROS) have been implicated in a wide variety of degenerative diseases including cancer, cardiovascular diseases, diabetes and its complications [7]. Growing evidence indicated that oxidative stress increases during diabetes due to overproduction of ROS and decreased efficiency of antioxidant defenses which starts very early and worsens over the course of disease [15]. During the development of diabetes, oxidation of lipids, proteins and DNA increases with time [8].

Implication of oxidative stress in the pathogenesis of diabetes is suggested due to non-enzymatic protein glycosylation, auto-oxidation of glucose, impaired glutathione metabolism, alteration in antioxidant enzymes, lipid peroxides formation and decreased ascorbic acid levels. The antioxidant enzymes *viz.* superoxide dismutase (SOD), glutathione peroxidase (GPxs) and catalase (CAT) contribute to eliminate superoxides, hydrogen peroxide and hydroxyl radicals [9-14]. Considering the fact that oxidative stress contributes towards T2DM and its complications, the present study was undertaken to assess the levels of antioxidant enzymes in T2DM patients in a north Indian population.

MATERIALS AND METHODS

Selection of subjects and collection of samples:

The study protocol was approved by the institutional ethical committee of King George's Medical University (KGMU) Lucknow, India and the study was performed during January-July 2012. Written informed consent was obtained from all participants. Blood was collected randomly from 474 T2DM patients (212 males and 262 females) and 301 control individuals (176 males and 125 females) were collected from the Diabetic Clinic and Pathology of Balrampur Hospital,

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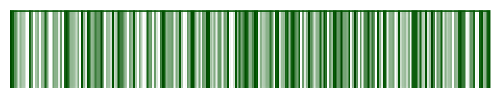

Lucknow respectively under the expert supervision of clinicians. Age of all diabetic and control subjects selected was within 20-70 years of age. The inclusion and exclusion criteria for controls and diabetic patients were according to National Diabetes Data Group (NDDG) and World Health Organization (WHO).

Four millilitre (ml) of peripheral blood collected was equally distributed in 0.5 M EDTA (pH- 8.0) and plain vials for enzyme assays and biochemical estimations respectively. Blood in plain vials were kept on ice for 30-60 min for separation of serum. Thereafter, serum was collected in fresh vials after centrifugation for 10 min at 3000 rpm and 4°C.

Anthropometric and Biochemical analysis

Body mass index (BMI) was calculated from weight in kilograms divided by height in meter square (kg/m^2). Mean systolic (SBP) and diastolic (DBP) blood pressures (disappearance of korotkoff sound phase V) were measured in the sitting position with an appropriately sized cuff after 5 min rest. Total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL), very low density lipoprotein (VLDL), high density lipoprotein (HDL), and serum creatinine (S CRET) levels were estimated by commercially available Ecoline kits (Merck, Germany).

Preparation of erythrocyte lysates

The EDTA blood was centrifuged at 2789 rpm (1000 xg) for 10 minutes at 4°C. The upper yellow plasma was taken out without disturbing the white buffy layer of leukocytes. Plasma was stored at –80°C for biochemical tests while the white buffy layer was discarded. The remaining lower layer contained erythrocytes (red blood cells) which were lysed with four times its volume of ice-cold HPLC-grade water and centrifuged at 10,000 rpm (12857 xg) for 15 minutes at 4°C. The supernatant was used for different antioxidant enzyme assays. Protein content was estimated in blood lysate by Biuret method [17].

Antioxidant enzyme assays:

Glutathione S transferase assay: The assay was performed by measuring the formation of a conjugate of glutathione and 1-chloro-2, 4, dinitro benzene (CDNB) according to the method described by Habig *et al* [18]. According to this method, reaction mixture containing 20 mM CDNB, 100 mM reduced glutathione (GSH), GST assay buffer (100mM Potassium phosphate buffer adjusted to pH 6.5, 0.1% TritonX-100) and 20 µl blood lysate was prepared and mixed thoroughly. CDNB was incubated at 37°C for 10 minutes before use and absorbance was taken at 340nm. The molar extinction coefficient of CDNB is 0.0096 $\mu M^{-1} cm^{-1}$.

Superoxide dismutase assay: SOD activity was measured by spectrophotometric method with minor modifications [19, 20]. Two reaction setups *viz.* experimental and reference were run in parallel. The experimental tubes contained 0.2 ml Nitro blue tetrazolium (NBT), 0.2 ml Phenozine methosulphate (PMS), 1.1 ml sodium pyrophosphate buffer and 20 µl enzyme sources while reference tubes had all reagents except the enzyme source. Both the reactions started simultaneously after addition of 0.2 ml nicotine adenine dinucleotide, reduced (NADH). After 90 seconds, 1.0 ml glacial acetic acid was added to each tube to stop the reaction and 20 µl enzyme sources was added to reference tubes. The absorbance was read at 560 nm. The difference in A_{560} between reference and an experimental reaction was the inhibition of NBT reduction by enzyme source. The SOD enzyme activity was defined as the amount of enzyme causing half the maximum inhibition of NBT reduction and was expressed as units/mg protein.

Catalase assay: Catalase activity was measured by spectrophotometric method as described by Aebi *et al* [21] with minor modifications [19], the decomposition of H_2O_2 was determined at 240 nm. According to this method, the reaction mixture containing 0.01M phosphate buffer (pH-7.0), 0.02M H_2O_2 and 20 µl blood lysate were mixed thoroughly and absorbance was taken at 240 nm after every 30 secs for 3 min. One catalase unit was defined as the amount of enzyme that decomposed 1 µmol of H_2O_2 per min at 37°C. The results were expressed as units/mg protein.

Statistical analysis:

Statistical analysis was performed by Prism software (v.5.01). Comparison of various groups was performed using the Students' t-test. All data were expressed as Mean ± SD. *P* <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

T2DM individuals are associated with 2 to 4 folds higher risk of cardiovascular disease compared with non-diabetics. This can be partly attributed to insulin resistance and dyslipidemia. The most common biochemical abnormalities in T2DM are TC, TG, LDL, VLDL, HDL and S CRET. In our study, results showed significant difference between SBP of normal and T2DM patients (P<0.001) while DBP did not show any significant change (P< 0.25). The mean value of post prandial sugar (268.8±5.266 mg/dl) and TC/LDL were significantly higher in patients with T2DM as compared to normal healthy subjects (P<0.0001), while that of HDL/VLDL was significantly decreased. No significant difference was observed in age and S CRET levels, however, BMI showed slight difference between T2DM and normal subjects (Table.1).

Table.1: Clinical characteristics of normal healthy subjects and T2DM patients (N, Number of samples)

Parameters	Normal Subjects (N=301)	Diabetic subjects (N=474)	P-value
Age (years)	49.48±0.34	49.21±0.44	0.68
BMI (kg/m^2)	23.63±0.07	24.39±4.53	0.0043
Systolic blood pressure (SBP) (mmHg)	120.2±13.57	135.47±19.72	<0.001
Diastolic blood pressure (DBP) (mmHg)	82.67±13.58	84.63±10.82	0.25
Post Prandial Sugar (PP)	126.8±10.97	268.8±5.266	< 0.0001
Total Cholesterol (TC) (mg/dl)	142.6±27.32	212.3±1.903	< 0.0001
Triglycerides (TG) (mg/dl)	134.1±27.32	114.4±0.9589	< 0.0001
Low density lipoprotein (LDL) (mg/dl)	54.86±29.93	140.4±2.378	< 0.0001
Very Low density lipoprotein (VLDL) (mg/dl)	26.83±12.04	22.89±0.1971	< 0.0001
High density lipoprotein (HDL) (mg/dl)	60.90±12.24	45.41±0.3733	< 0.0001
Serum Creatinine (S CRET) (mg/dl)	1.029±0.1116	1.05±0.109	0.60

Table.2: Comparison of antioxidant enzyme activities between normal healthy subjects and T2DM patients

Antioxidant enzymes	Normal		T2DM patients		P-value
	Number of samples	Mean±SE	Number of samples	Mean±SE	
GST (units/mg protein)	32	45.11±3.581	27	30.43±3.038	0.002
SOD (units/mg protein)	43	23.87±1.478	53	16.66±1.898	0.005
CAT (units/mg protein)	86	12.12±1.212	53	15.56±1.534	0.081

Table.3: Antioxidant enzyme activities in different age groups of normal healthy subjects and T2DM patients

Age group (y)	Total Sample	GST (units/mg protein) Mean ± SE	P-value	SOD (units/mg protein) Mean ± SE	P-value	CAT (units/mg protein) Mean ± SE	P-value
20 – 40	Normal	63.07 ± 1.593	< 0.0001	30.06 ± 2.026	0.3810	18.73 ± 1.295	0.0001
	Diabetic	30.91 ± 1.630		27.04 ± 2.261		7.866 ± 1.494	
41 – 60	Normal	49.10 ± 1.924	< 0.0001	19.01 ± 1.497	0.2030	16.86 ± 1.258	0.6430
	Diabetic	30.68 ± 1.815		17.04 ± 1.877		16.81 ± 2.406	
>60	Normal	35.43 ± 3.280	0.0061	16.87 ± 1.087	0.1775	18.50 ± 3.847	0.0753
	Diabetic	18.58 ± 1.613		14.94 ± 2.018		19.07 ± 1.045	

The erythrocytes are at increased risk due to oxidative processes for a variety of reasons. They are continually exposed to high oxygen tension. Hb is susceptible to autoxidation and can function as an oxidase and a peroxidase [22-24]. Therefore, the erythrocytes are completely dependent on the antioxidant defensive components throughout their 120 days of life span. These components build up an efficient defense system, an important part of which consists of three major enzymes: superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) [25]. The easy accessibility, finite life span and relative simplicity of erythrocytes make them an attractive model to study the oxidative stress status of the body in T2DM.

Free radicals are difficult to measure since they are unstable due to high reactivity and short life span. In the early stages of T2DM, the antioxidant defense system counters the effects of increased free radicals, but with advancement of disease, balance between generation of free radicals and antioxidant defense is impaired. In clinical states, the antioxidant levels or activity are determined by influence on other molecules or their mechanisms of action. Therefore, it is reliable to measure the activity levels of these antioxidant molecules.

The evaluation of antioxidant enzymes *viz.* SOD, CAT and GST in T2DM patients and normal subjects in our population indicated impaired antioxidant status in diabetic patients which is a definite sign of oxidative stress. We found statistically significant decrease in the

SOD and GST activities in patients when compared to controls (P=0.005, 0.002) (Table 2).

Comparing GST levels in different age groups (20-40, 40-60, < 60 years) of diabetic and normal subjects using ANOVA showed statistically significant association (P<0.0001, <0.0001, <0.0061 respectively) (Table.3; Fig. 1).

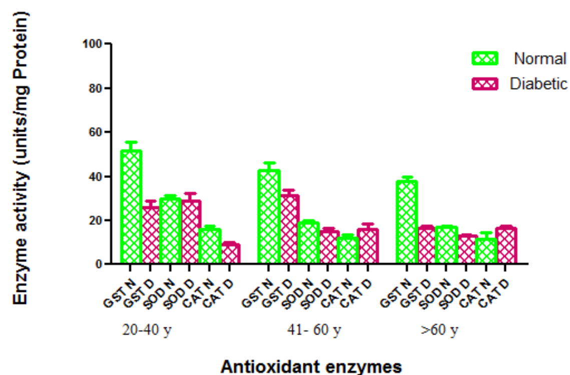

Fig.1: Antioxidant enzyme activities in different age groups of normal healthy subjects and T2DM patients (N, Normal; D, Diabetic; y, years).

Evidences also suggested that erythrocyte GST tends to decrease in diabetic patients [26] but there are also studies which show increased GST activity in diabetics independent of their glycemic status [27]. Decreased red blood cell GSH as well as increased serum total GST levels may be due to a compensatory mechanism of antioxidants to combat the oxidative stress in diabetic conditions with or without complications. The regulation of GST is subject to a complex set of endogenous and exogenous parameters. These include developmental, gender, and tissue-specific factors as well as a large number of xenobiotic-inducing agents. According to our study, GST levels were significantly higher in females than males in both groups (Fig. 2). Individual analysis of T2DM females and males with respect to controls showed significant decrease (P<0.0213, P<0.0001 respectively) (Table 4).

Table.4: Antioxidant enzyme activities in normal and diabetic males and females

Antioxidant enzymes		Males			Females		
		Number of samples	Mean ± SE (units/mg protein)	P-value	Number of samples	Mean ± SE (units/mg protein)	P-value
GST (units/mg protein)	Normal	16	30.08 ± 5.702	< 0.0001	12	42.88 ± 15.04	0.0213
	Diabetic	16	3.415 ± 0.421		19	18.38 ± 6.794	
SOD (units/mg protein)	Normal	14	22.04 ± 2.712	0.5432	24	25.02 ± 2.067	0.0002
	Diabetic	32	19.24 ± 2.774		18	12.72 ± 2.164	
CAT (units/mg protein)	Normal	45	12.93 ± 1.877	0.6993	39	11.18 ± 1.547	0.0467
	Diabetic	32	14.01 ± 1.962		18	17.16 ± 2.735	

Antioxidant enzyme SOD did not show significant association between the studied age groups whereas the overall activity showed significantly lower levels of the enzyme in T2DM when compared to normal subjects (P=0.005) (Table 2). When compared in males and females, SOD showed significant decrease only in female T2DM patients (P=0.0002) (Table 4). It was observed that decrease in GST was to a greater extent than SOD in male T2DM patients while both GST and SOD levels were low in female patients (Fig. 2).

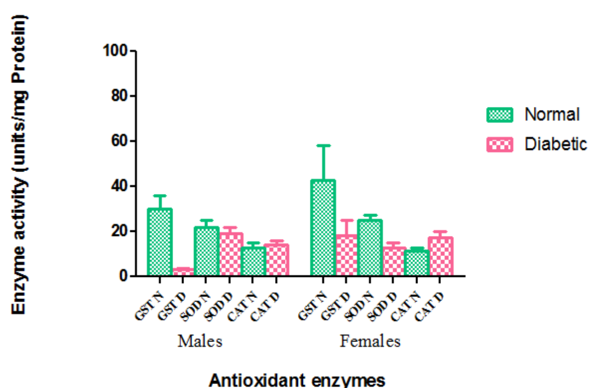

Fig 2: Graphical representation of antioxidant enzyme activities in normal and diabetic males and females (N, Normal; D, Diabetic).

Although not significant, there was a slight rise in CAT activity in T2DM patients when compared to healthy subjects (Table 2). Catalase is important in antioxidant defense against hydrogen peroxide [28,

29], but there are conflicting reports of decreases [30, 31], increases [32] and no change [33] in catalase activity under diabetic condition. There was a statistically significant difference in the level of catalase between diabetic and normal subjects with respect to 20- 40 age group, whereas no significant association was observed in other age groups (40-60, < 60 years) (Table 3). There was slight increase in CAT activity in diabetic condition both in males and females (Table 4, Fig. 2).

In our study, three broad age groups of T2DM patients, 20-40, 41-60 and >60 yrs were analyzed. When the activity levels of antioxidants was compared in different age groups, the enzyme activity of GST decreased significantly with age ($P< 0.0022$) while the decrease in SOD and catalase activities were not so significant (Table 3, Fig. 1). The activity of SOD decreases in erythrocytes due to aging or increase in glycation [34] depending on the decrease in electrolyte concentrations like copper and zinc which are parts of SOD enzyme [35, 36, 37]. Some studies have reported that sex or age has no affect on catalase activity [38], while other studies showed an increase or decrease in catalase activity with age [39]. Vitai and Goth [1997] showed that the activity was lower in healthy women in comparison to men, while the decreased activity by age was evident in men more than in women [40].

Ceballos *et al* [41] explained that during aging the steady state concentration of erythrocytic hydrogen peroxide (H_2O_2) may be much higher which could lead to enhanced peroxidation of poly unsaturated fatty acid (PUFA) present in cell membrane. This enhanced lipid peroxidation led to the lysis of erythrocytes resulting in an increase in levels of antioxidant enzymes GPx and CAT. It is known that free radicals play a role in beta cell damage [15, 42, 43]. SOD is the first line of defense against ROS which catalyzes dismutation of superoxide anion into H_2O_2, this is turn is transformed into oxygen and water by CAT and GPx. Besides H_2O_2, GPx also reduces lipid or non-lipid hydroperoxides while oxidizing glutathione (GSH). The oxidized GSH is then reduced by glutathione reductase (GR) [44]. Erythrocytes, which are equipped with a highly effective anti-oxidant defense system, can scavenge free radicals by the action of their antioxidant enzymes [45, 46]. In erythrocytes, glutathione detoxifies free radicals through GST conjunction. GST also possesses peroxidase activity and can directly attack the peroxides. Oxidative denaturation of hemoglobin (Hb) leads to the release of haemin into RBC membrane which is capable of oxidizing membrane proteins *via* a thiyl radical intermediate. GST can bind free haemin that is released during Hb oxidation presumably reducing damage to RBC membrane [47-49].

CONCLUSION

Our study in the Indian population showed that out of the three major antioxidant enzymes, GST and SOD levels were significantly lowered in T2DM patients as compared to normal healthy subjects. This means that the antioxidant enzyme production is affected in T2DM subjects leading to higher risk of cell organ damage. Therefore, detection of these antioxidant enzyme levels can be used as markers for monitoring T2DM in our population. This will enable the individuals at risk to take timely action and prevent or delay the onset of disease.

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