

## Original Article

# Role of Oxidative Stress, Antioxidant Enzymes, and TNF- $\alpha$ Levels in Diabetes Mellitus

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**ABSTRACT**

**Objective:** To study the relationship between glycemic control (as assessed by measurement of plasma glucose and glycosylated hemoglobin - HbA1c) and insulin levels with oxidative stress, tumor necrosis factor-alpha and antioxidant status in type 2 diabetes mellitus.

**Design:** Single measurement of the biochemical parameters in serum of diabetic patients and controls.

**Settings:** The outpatient clinic of the Amala Cancer Hospital, Kerala, India.

**Subjects:** Forty patients with type 2 diabetes mellitus (30 male, 10 female). Thirty patients had diabetes-induced complications, whereas 10 did not. Ten normal patients (8 male, 2 female) acted as controls.

**Main outcome measures:** Serum parameters of oxidative stress, antioxidant enzymes and tumor necrosis factor.

**Result:** The blood glucose and HbA1c levels of normal group were found to be heterogeneously significant ( $p$

$< 0.001$ ) in diabetic groups. There were no significant changes in the insulin levels between the two diabetic groups, while it was significantly low ( $p < 0.001$ ) in normal group. Significant elevation of malondialdehyde (MDA), hydroperoxide, and conjugated diene were found in both groups. The superoxidedismutase (SOD) and catalase levels were lower in complicated diabetic group. There were no significant alterations in the glutathione levels. Levels of glutathione peroxidase (GPx) and glutathione reductase (GR) were significantly low ( $p < 0.001$ ) in complicated diabetic group. Tumor necrosis factor- $\alpha$  was higher in both groups and the increase was significant ( $p < 0.001$ ) in the complicated diabetic group.

**Conclusion:** These results indicate that poor diabetic control is associated with increased oxidative stress and reduced antioxidant activity in type 2 diabetic patients.

KEY WORDS: antioxidant enzymes, glutathione, oxidative stress, tumor necrosis factor

**INTRODUCTION**

It is now known that oxygen at high concentrations can damage the liver, kidney, brain and other organs. The concept of biological free radicals is partially responsible for turning oxygen into a menace. Free radicals are a unstable and extremely reactive chemical species, which have an unpaired electron in their structure<sup>[1]</sup>. The most important free radicals are the radical derivatives of oxygen. Increased oxidative stress may result from over production of precursors to reactive oxygen radicals and/or decreased efficiency of inhibitory and scavenger systems. The stress, then may be amplified and propagated by an autocatalytic cycle producing tissue damage and cell death<sup>[2,3]</sup>. Cell damage will in turn, result in elevated production of reactive oxygen species (ROS). High levels of ROS have been found to play a role in the pathogenesis of type 2 diabetes mellitus. Altered antioxidant defenses in diabetes might lead to the development of diabetic induced complications.

Increased plasma level glucose is also responsible for the damage to cell membranes through non-enzymatic glycosylation of proteins, auto-oxidation of glucose and increased metabolism of glucose by the sorbitol-polyol pathway<sup>[4]</sup>.

The expression of tumor necrosis factor-alpha (TNF- $\alpha$ ), a cytokine secreted by macrophages and T cells was found to be increased in adipose tissue of obese animals and obese humans<sup>[5,6]</sup>. TNF- $\alpha$  blocks insulin receptor tyrosine kinase activity<sup>[7]</sup> and GLUT-4 expression<sup>[8]</sup> TNF- $\alpha$  has been shown to play a major role in the patho-biology of insulin resistance and development of type 2 diabetes mellitus .

The present study was undertaken to find out the role of oxidants and antioxidants in the pathogenesis of diabetes mellitus, especially in the case of diabetes related complications. We evaluated the enzymatic and non-enzymatic antioxidant activity in serum and erythrocytes of type 2 diabetes patients and compared it with normal persons. We have also

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tried to relate the oxidative status in diabetes with serum TNF- $\alpha$  levels.

## PATIENTS AND METHODS

Both newly detected and known diabetic subjects diagnosed for type 2 diabetes were enrolled, including those who were on anti-diabetic therapy. The diagnosis of diabetes was based on the guidelines recommended by the American Diabetes Association<sup>[9]</sup>.

Patients with type 2 diabetes mellitus (n = 40; 30 male and 10 female) were recruited from the Amala Hospital, Thrissur. All diabetic patients had undergone treatment with sulphonylureas (either glibenclamide or glipizide). Normal controls (n = 10; 8 male and 2 female) were recruited from Amala Cancer Research Centre. None of the study subjects was taking antioxidant vitamins at the time of blood sampling. Ten out of 30 in the complications groups had microvascular involvement (nephropathy). The other 20 complicated diabetic patients had microvascular complication (coronary artery disease). For experimental purpose they were grouped as follows. Group I - diabetic patients with complication (n = 30); Group II - diabetic patients without complications (n = 10); Group III - normal controls (n = 10).

Blood was taken from a forearm vein from the individuals according to protocol approved by the Institution Human Experiments Review Committee. One part was aliquot into ethylenediamine tetra acetic acid (EDTA)-containing tubes for the measurement of glycated hemoglobin (HbA1c) as well as enzymatic assays. Another part of the blood was collected separately, centrifuged at 1500 rpm x 20 minutes, serum aspirated and immediately stored at -20° C. The serum was used for glucose, TNF- $\alpha$ , lipid peroxidation (LPO), hydroperoxides and conjugated dienes estimations.

Insulin assay was done using the method of Morgan and Lazarow<sup>[10]</sup> with a radioimmunoassay kit provided by Board of Radiation and Isotope Technology, BARC, Mumbai, India. Blood glucose was determined by the glucose oxidase method<sup>[11]</sup>. TNF- $\alpha$  was determined by the ELISA method (Titerzyme-EIA).

Erythrocytes were prepared by the method of Minami and Yoshikawa<sup>[12]</sup> and superoxide dismutase (SOD) was estimated in erythrocytes by the modified method of McCord and Fridovich<sup>[13]</sup>. The assay is based on the ability of the enzyme to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide, which is generated by the photo reduction of riboflavin. One unit of enzyme activity is defined as amount of enzyme giving 50% inhibition of the reduction of NBT and expressed as units/g Hb.

Catalase activity in blood was determined by the method of Aebi<sup>[14]</sup>, by measuring the rate of decomposition of hydrogen peroxide at 240 nm. A decrease in absorbance was observed after the addition of H<sub>2</sub>O<sub>2</sub> to the reaction mixture containing the erythrocytes, which is used as the source of catalase. Units of activity were determined from the E<sub>max</sub> of H<sub>2</sub>O<sub>2</sub>. Reduced glutathione (GSH) activity in the blood was measured by the method of Moron<sup>[15]</sup>, based on the reaction with 5-5' dithiobis (2-nitrobenzoic acid). Values were calculated from a standard graph of GSH treated with the same reagent. Glutathione peroxidase (GPx) activity in blood was determined by the method of Paglia and Valentine<sup>[16]</sup> based on the degradation of hydrogen peroxide in the presence of reduced glutathione. Reduction of GSH concentration was determined by reacting with 5-5' dithiobis (2-nitrobenzoic acid) and values were calculated from a standard plot of GSH. The activity of glutathione reductase in blood was determined by Racker's method<sup>[17]</sup>, based on the amount of NADPH consumed during the conversion of oxidized glutathione to reduced glutathione. The decrease in absorbance/min was followed using 1 min intervals for 5 min at 340 nm and the concentrations were calculated from the E<sub>max</sub> of NADP. Lipid peroxidation in serum was done by the TBA method as modified by Ohkawa<sup>[18]</sup>, using thiobarbituric acid method. Hydroperoxides and conjugated dienes in erythrocytes were determined by the modified method of John and Steven<sup>[19]</sup>. In both tests, samples were first extracted in chloroform and methanol and the lower layer was taken to dryness. The remaining lipid residue was dissolved in 1.5 ml cyclohexane and the absorbance was taken at 233 nm. For the estimation of hydroperoxides, the lipid residues were treated with potassium iodide and cadmium acetate and the absorbance was then measured at 353 nm. Hemoglobin was estimated by the cyanmethemoglobin method using Drabkin's solutions<sup>[20]</sup> and protein was estimated by Lowry's method<sup>[21]</sup>.

Statistical calculations were performed using Star View soft ware package. All values are expressed as mean  $\pm$  SD. Comparisons of values between groups were made using one way ANOVA followed by Bartlett's test and p-value < 0.001 and < 0.05 were considered significant<sup>[22]</sup>.

## RESULTS

A total of 40 diabetic patients were studied, of which 30 were male and 10 were female. Their mean age was 53.35  $\pm$  4.98 years. The mean duration of diabetes was 9.16  $\pm$  5.64 years. The mean post prandial blood glucose level of diabetic patients was 233  $\pm$  102.29 mg%.

**Table 1:** Levels of post prandial serum glucose, HbA1c, TNF- $\alpha$  and insulin in normal and diabetic patients

Groups	Serum glucose (mg/dl)	HbA1c (%)	TNF- $\alpha$ (pg/ml)	Insulin (IU/ml)
I	254.30 <sup>a</sup> ± 107.61	15.47 <sup>a</sup> ± 5.30	127.27 <sup>a</sup> ± 58.79	21.80 <sup>a</sup> ± 16.15
II	170.20 <sup>b**</sup> ± 46.29	12.89 <sup>a</sup> ± 3.27	73.70 <sup>b**</sup> ± 21.99	27.00 <sup>a</sup> ± 13.72
III	112.21 <sup>c**</sup> ± 11.62	6.30 <sup>b**</sup> ± 1.06	30.70 <sup>b**</sup> ± 3.74	12.2 <sup>b*</sup> ± 2.83

Superscript indicate the significant between diabetic and normal groups. Means having different superscript are significant at \*\*p < 0.001, \*p < 0.05.

**Table 3:** Effect of diabetes on lipid peroxidation, hydroperoxide and conjugated diene in erythrocytes

Groups	LPO (n moles/ml)	HP (U/g Hb)	CD (U/g Hb)
Complicated diabetic	5.78 <sup>a</sup> ± 1.13	2.47 <sup>a</sup> ± 0.79	2.65 <sup>a</sup> ± 0.80
Uncomplicated diabetic	5.11 <sup>a</sup> ± 0.70	1.98 <sup>ab</sup> ± 0.63	2.17 <sup>a</sup> ± 0.48
Normal	4.04 <sup>b**</sup> ± 0.52	1.37 <sup>b**</sup> ± 0.36	1.45 <sup>b**</sup> ± 0.36

LPO: Lipid peroxidation; HP: Hydroperoxide; CD: Conjugated diene; Superscripts indicate the significance between diabetic and normal groups. Means having different superscripts are significant at \*\*p < 0.001, \*p < 0.05.

Thirty out of forty diabetics (75%) were suffering from one or more diabetic-induced vascular complications (nephropathy or coronary artery disease). The mean age of complicated diabetics (Group I) was 55.46 ± 3.47 years compared to a mean of 47 ± 2.98 years for the uncomplicated diabetics (Group II). The mean duration of diabetes in Group I was 11.58 ± 4.19 years compared to a mean of 1.80 ± 1.79 years in Group II. The mean postprandial blood glucose levels in Groups I and II were 254.32 ± 107.59 mg/dl and 170.20 ± 46.29 mg/dl respectively (Table 1).

The blood glucose levels of the normal control group (Group III) were heterogeneously significant (p < 0.001) with diabetic patients. Group I showed highest glucose level (254.30 ± 107.61) when compared Group II (70.20 ± 46.29) and Group III (112.21 ± 11.62) patients. The elevations of glycated hemoglobin levels were comparable with that of plasma glucose levels of the same group. Among the diabetic groups (I and II) HbA1c did not differ significantly but the difference was significant when compared to Group III (p < 0.001, Table 1).

TNF- $\alpha$  level in Group II patients significantly low (p < 0.001, 73.7 ± 21.99) when compared with Group I (127.27 ± 58.79). TNF- $\alpha$  level showed no significant difference between Group II and III. Group I showed a highly significant increase in

**Table 2:** Levels of antioxidant enzymes in normal and diabetic patients

Groups	SOD <sup>1</sup> (U/g Hb)	CATALASE <sup>2</sup> (k/g Hb)
Complicated diabetic	953.30 <sup>a</sup> ± 127.53	86.96 <sup>a</sup> ± 18.91
Uncomplicated diabetic	1050.53 <sup>a</sup> ± 193.84	93.69 <sup>a</sup> ± 16.21
Normal	1336.72 <sup>b*</sup> ± 231.06	109.07 <sup>b*</sup> ± 11.31

SOD - Superoxide dismutase

Superscripts indicate the significance between diabetic and normal groups. Means having different superscripts are significant at \*\*p < 0.001, \*p < 0.05.

**Table 4:** Levels of glutathione, glutathione peroxidase and glutathione reductase in erythrocytes of normal and diabetic patients

Groups	GSH (n moles/ml)	GPx (U/l)	GR (U/l)
Complicated diabetic	185.78 <sup>ac</sup> ± 25.12	15.15 <sup>a</sup> ± 2.98	7.82 <sup>a</sup> ± 1.17
Uncomplicated diabetic	197.74 <sup>ab**</sup> ± 14.39	17.60 <sup>b**</sup> ± 2.69	8.87 <sup>b**</sup> ± 0.78
Normal	197.40 <sup>bc**</sup> ± 28.63	19.82 <sup>b**</sup> ± 1.66	9.44 <sup>b**</sup> ± 0.65

GSH: Glutathione; GPx: Glutathione peroxidase; GR: Glutathione reductase. Superscripts indicate the significance between diabetic and normal groups. Means having different superscript are significant at \*\*p < 0.001, \*p < 0.05.

TNF- $\alpha$  levels when compared with Group III (p < 0.001, Table 1).

There were no significant changes in the insulin levels between Group I and II. However, Group II showed significant increase in the insulin levels as compared to Group III (p < 0.05). Even though the insulin levels were increased in Group I, the increase was not significant (Table 1).

The changes of *in vivo* antioxidant levels like superoxide dismutase and catalase are shown in Table 2. The SOD level was found to be significantly low in Groups I and II compared to Group III. The catalase levels did not show significant changes among the diabetic groups but were significantly higher in Group III (p < 0.05).

Lipid peroxidation, which is measured as the malondialdehyde formed (MDA), was not heterogeneously significant between the diabetic groups. However, the lipid peroxidation levels were significantly low in Group III as compared to Groups I and II (p < 0.001). Hydroperoxides and conjugated dienes were not significantly different in diabetic groups. Hydroperoxide levels in Group III were significantly low when compared to Group I (p < 0.001). The conjugated diene levels in diabetic patients were significantly higher than the control group (p < 0.001, Table 3).

Glutathione levels of diabetic patients as well as controls were not found to be significant. Glutathione peroxidase levels in Group I were significantly low when compared with Group II and III ( $p < 0.001$ ). Glutathione reductase levels in Group II and III were significantly higher when compared with Group I ( $p$ -value  $< 0.001$ , Table 4).

## DISCUSSION

In this study, we found that poor glycemic control in diabetic patients was associated with decreased free radical scavenging activity. In hyperglycemia, glucose undergoes auto-oxidation and produces free radicals that in turn lead to peroxidation of lipids in lipoproteins. Elevated levels of lipid peroxidation, hydroperoxide and conjugated diene seen in diabetic patients are clear manifestations of excessive formation of free radicals resulting in tissue damage. The activity of superoxide dismutase was found to be lower in diabetic patients when compared to normal. This decrease in SOD activity could result from inactivation and/or glycation of the enzyme, which are known to occur in diabetes<sup>[23]</sup>. GSH is a major nonprotein thiol in living organisms and plays a central role in coordinating the body's antioxidant defense processes. Perturbation of GSH status of a biological system can lead to serious consequences. In our study we could not find any difference in the blood GSH levels of normal and diabetic patients. However, many of the enzymes related to the GSH protein have been found to be lowered in diabetes. GPx catalyses the reaction of hydroperoxides and reduce glutathione to form glutathione disulphide (GSSG). GPx levels were found to be lowered in diabetic patients. Glutathione reductase, which reduces oxidized glutathione, was also lower in diabetics. Similarly catalase which reduces  $H_2O_2$  has been found to be significantly lowered in diabetic patients when compared with normal and it was comparable with earlier results<sup>[24]</sup>.

Long-term vascular complications still represent the main cause of morbidity and mortality in diabetic patients. Although prospective randomized long-term clinical studies comparing the effects of conventional and intensive therapy have demonstrated a clear link between diabetic hyperglycemia and the development of secondary complications of diabetes, they have not defined the mechanism through which excess glucose results in tissue damage. Evidence has accumulated indicating that oxidative stress may play an important role in the etiology of diabetic complications. This hypothesis is supported by the evidence that many biochemical pathways associated with hyperglycemia such as glucose autoxidation, polyol pathway, prostanoid synthesis and protein glycation are the result of the

increased production of free radicals<sup>[25]</sup>.

In our study we have observed that TNF- $\alpha$  levels increased significantly in many diabetic patients who had complications. TNF- $\alpha$  has been shown to have a critical role in insulin resistance. In septic shock the TNF- $\alpha$  level is increased and resistance to insulin has been reported<sup>[26]</sup>. Exposure to TNF- $\alpha$  has been shown to decrease GLUT-4 protein in cultured cells producing a decrease in the glucose transport. It has been shown that TNF- $\alpha$  blocks the insulin receptor tyrosine kinase activity<sup>[27]</sup>.

## CONCLUSION

Herbal extracts are reported to be useful in diabetic conditions<sup>[28]</sup> and the exact roles of these extracts have not been identified. We have reported earlier that many of the plants extracts also have significant antioxidant activity, which has been shown to reduce glucose level during diabetic conditions<sup>[29]</sup>. Increased TNF- $\alpha$  levels in alloxan diabetic animals were found to be decreased by the plant extracts (Sabu & Kuttan, unpublished) indicating that herbal extracts could inhibit TNF- $\alpha$  levels. This may be another mechanism of their action. Antioxidants and TNF- $\alpha$  inhibitors may thus be important in the control of diabetes and its long-term complications. In the present study diabetic patients with increased TNF- $\alpha$  were found to have increased insulin levels when compared to normal controls, which in turn can produce insulin resistance.

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