Chapter 5

Oxidative stress in Type 2 diabetic patients
5.1 INTRODUCTION

It is now known that oxygen at high concentrations can damage the liver, kidney, brain and other organs. Biological free radicals are partially responsible for turning oxygen into a menace. Free radicals are an unstable and extremely reactive chemical species, which have an unpaired electron in their structure [Dormandy, 1980]. 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 produces tissue damage and cell death [Brownlee et al., 1988; Hunt et al., 1993]. Cell damage will in turn; result in elevated production of reactive oxygen species or ROS. High levels of ROS have been found to play a role in the pathogenesis of NIDDM. Altered antioxidants 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 [Sato et al., 1979].

The expression of tumour necrosis factor- alpha (TNF- α), a cytokine secreted by macrophages and T cells was found to be increased in adipose tissue of obese animals and obese humans [Hotamisligil et al., 1993; Saghizadeh et al., 1996]. TNF-alpha blocks insulin receptor tyrosine kinase activity [Feinsteine, 1993] and GLUT-4 expression [Stephens, 1997]. TNF-alpha has been shown to play a major role in the patho-biology of insulin resistance and development of NIDDM.

The present study was undertaken to find out the role of oxidants and antioxidants in the pathogenesis of human diabetes mellitus, especially during secondary complications. We evaluated the enzymatic and non-enzymatic antioxidant activity in serum and erythrocytes of
non-insulin dependent diabetic (NIDDM) patients and compared with normal persons. We have also tried to relate the oxidative status in diabetes with serum TNF-alpha levels.

5.2 PATIENTS AND METHODS

Newly detected and old NIDDM diabetic cases 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 (July 97) [Gavin, 1997].

Patients with non-insulin dependent (NIDDM) diabetes mellitus (n=40; 30 males and 10 females) were recruited from the Amala Hospital, Thrissur, while normals (n=10; 8 males and 2 females) were recruited from Amala Cancer Research Centre. None of the study subjects was taking antioxidant vitamins at the time of blood sampling. They were grouped under three categories. Group I – diabetic patients with complications (nos. 30); Group II – diabetic patients without complications (nos.10); Group III – normals (nos.10).

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

Insulin assay was done with a radioimmunoassay kit provided by Board of Radiation and Isotope Technology, BARC, Mumbai, India by the method of Morgan and Lazarow [Morgan and Lazarow, 1965]. Blood glucose was determined by the glucose oxidase method [Trinder, 1969]. Tumour necrosis factor (TNF-α) was determined by the ELISA method (Titerzyme-EIA).
Erythrocytes were prepared by the method of Minami and Yoshikawa, [1979] and superoxide dismutase (SOD) was estimated in erythrocytes and liver by the modified method of McCord and Fridovich, [1969]. 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, [1974], by measuring the rate of decomposition of hydrogen peroxide at 240 nm. A decrease in absorbance was observed after the addition of H$_2$O$_2$ to the reaction mixture containing the erythrocytes, which is used as the source of catalase. Units of activity were determined from the $E_{max}$ of H$_2$O$_2$. Reduced glutathione (GSH) activity in the blood was measured by the method of Moron et al., [1979] 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, [1967] 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 [1955], 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 5min at 340 nm and the concentrations were calculated from the $E_{max}$ of NADP. Lipid peroxidation in serum was done by the TBA method as modified by Ohkawa et al., [1979] using thiobarbituric acid method. Hydroperoxides and conjugated dienes in erythrocytes were determined by the modified
method of John and Steven, [1978]. 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. Haemoglobin was estimated by the cyanmethaemoglobin method using Drabkin’s solutions [Carman, 1993] and protein was estimated by Lowry’s method [Lowry et al., 1951].

5.2.1 Statistical analysis

Statistical calculations were performed using Star View software package. All values are expressed as mean ± SD. Comparisons of values between groups were made using one way ANOVA followed by Bartlett’s test and values with p<0.001, p<0.05 were considered significant [Snedecor and Cochran, 1967].

5.3 RESULTS

A total of 40 diabetic patients were studied, out of which 30 were males and 10 were females. The mean age of the study was 53.35 ± 4.98 years. The mean duration of diabetes was 9.16 ± 5.64 years. The mean post-prandial blood sugar level of diabetic patients was 233 ± 102.29.

Thirty out of forty-diabetics (i.e. 75 %) were suffering from one or more diabetic-induced complications. The mean age of complicated diabetics was 55.46 ± 3.47 years compared to a mean of 47 ± 2.98 years of the uncomplicated diabetics. The mean duration of complicated diabetics was 11.58 ± 4.19 years compared to a mean of 1.80 ± 1.79 years of the uncomplicated diabetics. The mean post-prandial blood sugar levels of uncomplicated and complicated diabetics were 170.20 ± 46.29 mg/dl and 254.32 ± 107.59 mg/dl respectively (Table 5.1).

The blood glucose levels of normal groups were heterogeneously significant (p<0.001) with diabetic patients. The complicated diabetic patients showed highest glucose level (254.30±107.61) when compared
Table 5.1 Levels of post prandial serum glucose, HbA1c, TNF-α and insulin in normal and diabetic patients.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Plasma glucose</th>
<th>HbA1c</th>
<th>TNF-α</th>
<th>Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complicated</td>
<td>254.30^A</td>
<td>15.47^A</td>
<td>127.27^A</td>
<td>21.80^A</td>
</tr>
<tr>
<td>Diabetic</td>
<td>±107.61</td>
<td>±5.30</td>
<td>±58.79</td>
<td>±16.15</td>
</tr>
<tr>
<td>Uncomplicated</td>
<td>170.20^B**</td>
<td>12.89^A</td>
<td>73.70^B**</td>
<td>27.00^A</td>
</tr>
<tr>
<td>Diabetic</td>
<td>±46.29</td>
<td>±3.27</td>
<td>±21.99</td>
<td>±13.72</td>
</tr>
<tr>
<td>Normal</td>
<td>112.21^C**</td>
<td>6.30^B**</td>
<td>30.70^C**</td>
<td>12.2^B*</td>
</tr>
<tr>
<td></td>
<td>±11.62</td>
<td>±1.06</td>
<td>±3.74</td>
<td>±2.83</td>
</tr>
</tbody>
</table>

Units: 1- mg/dl; 2- percentage; 3- pg/ml; 4- IU/ml. Values are mean ± SD.; n=6.

Capital letters indicate comparison between diabetic and normal groups. Means having different capital letters are significant at **p<0.001, *p<0.05, but the same letters are not significant.
with uncomplicated (170.20 ±46.29) and normal (112.21±11.62) groups. The elevations of glycated hemoglobin levels were comparable with that of plasma glucose levels of the same group. Among the diabetic groups HbA1c did not show significant difference between these groups but were heterogeneously significant (p<0.001) with that of the normal (Table 5.1).

Tumour necrosis factor-α level of uncomplicated diabetes was significantly (p<0.001) low (73.7±21.99) when compared with complicated diabetes (127.27 ±58.79). TNF-α level showed less significant difference (p<0.05) between normal and uncomplicated diabetic group. Complicated diabetic group showed highly significant increase (p<0.001) in TNF-α levels when compared with normals (Table 5.1).

There was no significant change in the insulin levels between the diabetic groups. When compared with normal group, the uncomplicated diabetic patients showed significant (p<0.05) increase in the insulin levels. Even though the insulin levels were increased in complicated diabetic, the increase was not significant (Table 5.1).

The changes of in vivo antioxidant levels like superoxide dismutase and catalase were given in Table 5.2. The SOD level was found to be significantly low in both diabetic groups compared to normals. The catalase levels did not show significant changes among the diabetic groups where as, the catalase levels were significantly (p<0.05) higher in normal when compared with diabetic groups.

Lipid peroxidation, which is measured as the malondialdehyde formed (MDA), was not heterogeneously significant between the diabetic groups. Lipid peroxidation levels were significantly (p<0.001) low in normals when compared with both diabetic groups. Hydroperoxides and conjugated dienes were not significantly different in diabetic groups. Hydroperoxide levels of normals were significantly low compared to complicated diabetic group (p<0.001). The conjugated diene levels of
Table 5.2 Levels of antioxidant enzymes in normal and diabetic patients

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD$^1$</th>
<th>CATALASE$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complicated</td>
<td>953.30$^A$</td>
<td>86.96$^A$</td>
</tr>
<tr>
<td>Diabetic</td>
<td>±127.53</td>
<td>±18.91</td>
</tr>
<tr>
<td>Uncomplicated</td>
<td>1050.53$^A$</td>
<td>93.69$^A$</td>
</tr>
<tr>
<td>Diabetic</td>
<td>±193.84</td>
<td>±16.21</td>
</tr>
<tr>
<td>Normal</td>
<td>1336.72$^B*$</td>
<td>109.07$^B*$</td>
</tr>
<tr>
<td></td>
<td>±231.66</td>
<td>±11.31</td>
</tr>
</tbody>
</table>

SOD-Superoxide dismutase; Units: 1-U/g Hb; 2-k/g Hb. Values are mean ± SD.; n=6
Capital letters indicate comparison between diabetic and normal groups. Means having different capital letters are significant at *p<0.05, but the same letters are not significant.
Table 5.3 Effect of diabetes on lipid peroxidation, hydroperoxide and conjugated diene in erythrocytes.

<table>
<thead>
<tr>
<th>Groups</th>
<th>LPO$^1$</th>
<th>HP$^2$</th>
<th>CD$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complicated</td>
<td>5.78A</td>
<td>2.47A</td>
<td>2.65A</td>
</tr>
<tr>
<td>Diabetic</td>
<td>±1.13</td>
<td>±0.79</td>
<td>±0.80</td>
</tr>
<tr>
<td>Uncomplicated</td>
<td>5.11A</td>
<td>1.98AB</td>
<td>2.17A</td>
</tr>
<tr>
<td>Diabetic</td>
<td>±0.70</td>
<td>±0.63</td>
<td>±0.48</td>
</tr>
<tr>
<td>Normal</td>
<td>4.04B**</td>
<td>1.37B**</td>
<td>1.45B**</td>
</tr>
<tr>
<td></td>
<td>±0.52</td>
<td>±0.36</td>
<td>±0.36</td>
</tr>
</tbody>
</table>

LPO-Lipid peroxidation, HP- Hydroperoxide, CD- Conjugated diene

Units: 1- n moles/ml; 2- U/g Hb; 3- U/g Hb. Values are mean ± SD.; n=6.

Capital letters indicate comparison between diabetic and normal groups. Means having different capital letters are significant at **p<0.001, but the same letters are not significant.
Table 5.4 Levels of glutathione, glutathione peroxidase and glutathione reductase in erythrocytes of normal and diabetic patients.

<table>
<thead>
<tr>
<th>Groups</th>
<th>GSH$^1$</th>
<th>GPx$^2$</th>
<th>GR$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complicated diabetic</td>
<td>$185.78^{AC}$</td>
<td>$15.15^A$</td>
<td>$7.82^A$</td>
</tr>
<tr>
<td></td>
<td>$\pm 25.12$</td>
<td>$\pm 2.98$</td>
<td>$\pm 1.17$</td>
</tr>
<tr>
<td>Uncomplicated diabetic</td>
<td>$197.74^{AB**}$</td>
<td>$17.60^{B**}$</td>
<td>$8.87^{B**}$</td>
</tr>
<tr>
<td></td>
<td>$\pm 14.39$</td>
<td>$\pm 2.69$</td>
<td>$\pm 0.78$</td>
</tr>
<tr>
<td>Normal</td>
<td>$197.40^{BC**}$</td>
<td>$19.82^{B**}$</td>
<td>$9.44^{B**}$</td>
</tr>
<tr>
<td></td>
<td>$\pm 28.63$</td>
<td>$\pm 1.66$</td>
<td>$\pm 0.65$</td>
</tr>
</tbody>
</table>

GSH- Glutathione; GPx – Glutathione peroxide; GR – Glutathione reductase

Units: 1-n moles/ml; 2- U/l; 3 – U/l, Values are mean ± SD.; n=6.

Capital letters indicate comparison between diabetic and normal groups. Means having different capital letters are significant at **p<0.001, but the same letters are not significant.
diabetic patients were significantly (p<0.001) higher than that of normal
(Table 5.3).

Glutathione levels of diabetic patients as well as normals were not
found to be significant. Glutathione peroxidase levels of complicated
diabetic patients were significantly (p<0.001) low when compared with
uncomplicated diabetic patients and normals. Glutathione reductase of
normals and uncomplicated diabetes were significantly (p<0.001) higher
when compared with complicated diabetes (Table 5.4).

5.4 DISCUSSION

The pathogenesis of diabetes mellitus has been a subject of
controversy and exact mechanism for its development has remained
unclear. In our study of 40 diabetic patients, majority were males (75 %).
There was a wide variation in the duration of diabetes, with the mean
duration of the complicated diabetic sub group significantly higher than
their uncomplicated counterparts. Most of the patients in this group had
poor glycemic control. Seventy-five percentages of these patients were
suffering from at least one-micro/macrovascular complications. The
duration of diabetes is said to be an important predictor for the
development of vascular complications [Sud and Sakhuja, 1997; Pirart,
1978; Klein et al., 1989].

Evidence has accumulated indicating that the generation of
reactive oxygen species (Oxidative stress) may play an important role in
the etiology of diabetic complications. In this study, we have found that
poor glycaemic control in diabetic patients was also associated with
decreased free radical scavenging activity. In hyperglycaemia, glucose
undergoes auto-oxidation and produces free radicals that in turn leads to
peroxidation of lipids in lipoproteins. Elevated levels of lipid peroxidation,
hydroperoxide and conjugated diene seen in diabetic patients are clear
manifestation 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 of the enzyme by $\text{H}_2\text{O}_2$ or by glycation of the enzyme, which are known to occur during diabetes [Skrha et al., 1996]. GSH is a major non-protein thiol in living organisms, which 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 the 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 has been found to be lowered in diabetes. GPx catalyses the reaction of hydroperoxides and reduces 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 during diabetes. Hence it is postulated that the levels of GSH may be lowered in the tissues during diabetes. Similarly catalase, which reduces $\text{H}_2\text{O}_2$, has been found to be significantly low in diabetic patients [Baynes, 1991].

The results of this study suggest that poor glycaemic control is associated with reduced serum free radical scavenging activity among patients with NIDDM. Glycated haemoglobin is recognized as a good measure of long term glycaemic control giving information about the integrated mean blood glucose concentration over the preceding 3-6 weeks. This association may reflect increased free radical production. Persistent hyperglycaemia promotes non-enzymatic glycation of proteins and local free radical production as glucose undergoes autoxidation in situ [Hunt et al., 1988; Azevedo et al., 1988]. The glycation end-products produced by these redox reactions are involved in the development of vascular complications [Brownlee et al., 1988]. Free glucose can also auto-oxidise under physiological conditions leading to production of alpha-ketoaldehydes and other oxidizing intermediates [Wolff and Dean, 1987]. For these reasons, persistent hyperglycaemia may expose the diabetic to increased free radical stress and might be expected to reduce antioxidant reserves. This sequence of events has been previously
suggested but there has been little evidence to support a relationship between antioxidant depletion and glycaemic control. Increased free radical generation and hyperglycaemia may indirectly impair the regeneration of antioxidant molecules. Excessive intracellular delivery of glucose stimulates the activity of the enzyme aldose reductase, which reduces glucose to sorbitol. This enzyme requires the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) as a co-factor. Consequently, the availability of NADPH for the intracellular regeneration of antioxidant molecules such as glutathione or vitamin C is limited [Collier and Small, 1991]. A rational extension of this proposed role for oxidative stress is the suggestion that the different susceptibility of diabetic patients to microvascular and macrovascular complications may be a function of the endogenous antioxidant status.

The inverse relationship of glycated haemoglobin and total antioxidant activity could also result from the direct inhibition of protein glycation by serum antioxidants. In vitro studies support this possibility. Antioxidants such as vitamin C and vitamin E inhibit glucose autoxidation and reduce the covalent linking of glucose to serum proteins [Davie et al., 1992; Ceriello et al., 1998]. Furthermore, studies in vitro also suggested that dietary antioxidant supplementation can inhibit the glycation of serum proteins [Ceriello et al., 1991].

There is complex relationship between TNF-α and diabetic complications. In our study we have observed that TNF-α levels increased significantly in many diabetic patients who had complications. TNF-α related apoptosis-inducing ligand is involved in β cell damage leading to diabetes, causes insulin resistance, and interferes with insulin signaling and influence the formation of atherosclerotic vascular lesions in diabetic patients [Hotamisligil, 1999]. Increased TNF-α levels in patients with diabetes has been demonstrated and determination of TNF-α levels might offer a diagnostic tool to determine patients at high risk [Lamhamed-Cherradi et al., 2003]. Exposure to TNF-α has been shown to decrease
GLUT-4 protein in cultured cells producing a decrease in the glucose transport. It has been shown that TNF-α blocks the insulin receptor tyrosin kinase activity [White and Kahn, 1994].

Herbal extracts have reported to be useful in diabetic conditions [Sabu et al., 2002]. The exact roles of these extracts have not been identified. We have reported earlier that many of these extracts also have significant antioxidant activity, which has been shown to be reduced during diabetic conditions [Sabu and Kuttan, 2002]. Increased TNF-α levels in alloxan diabetic animals were found to be decreased by the plant extracts (Chapter 6) indicating that herbal extracts could inhibit TNF-α levels and this may be another mechanism of their action. Antioxidants and TNF-α inhibitors may thus be important in the control of diabetes and its long-term complications.

In the present study diabetic patients with increased TNF-α were found to have increased insulin levels when compared to normal. Increased insulin production could attribute to the insulin resistance seen in the diabetic patients.