Antioxidants and Lipid Peroxidation Status in Diabetic Patients with and without Complications

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Asha Kamath MPhil***, Anjali Rao PhD•**

Background: Oxidative stress is involved in the pathophysiology of diabetes mellitus.

Methods: In the present study, 68 patients with type 2 diabetes mellitus and 31 clinically healthy individuals were evaluated. The patients were divided into two groups. Group 1 included 29 patients without diabetic complications and group 2 consisted of 39 patients with diabetic complications. Erythrocyte glutathione, superoxide dismutase, and thiobarbituric acid-reactive substance levels as well as plasma antioxidant vitamins C and E, and serum total glutathione-S-transferase, ceruloplasmin, and protein thiols were estimated by using spectrophotometer.

Results: A significant decrease of erythrocyte glutathione was observed in group 1 when compared with the controls. Thiols decreased in group 2. An increase in glutathione-S-transferase, ceruloplasmin, superoxide dismutase, and vitamins C and E levels was noted in patients with diabetes mellitus. Thio-barbituric acid-reactive substance levels decreased in group 1 but increased in group 2 when compared with the controls.

Conclusion: In the present study, tendency of most of the antioxidants to rise in diabetes could probably be due to an adaptive response to the pro-oxidant milieu of the diabetic state. Hence, we suggest that supplementation with dietary antioxidants especially antioxidant vitamins accompanied by change in lifestyle might help to reduce damage brought about by free radical toxicity in diabetes mellitus.

Introduction

Diabetes mellitus (DM) in all its heterogeneity has taken the center stage as one of the ultimate medical challenges. Diabetic complications are the major cause of morbidity and mortality in patients with DM. Chronic hyperglycemia is a major initiator of diabetic microvascular complications (e.g., retinopathy, neuropathy, and nephropathy). Glucose processing uses a variety of diverse metabolic pathways; hence, chronic hyperglycemia can induce multiple cellular changes leading to complications.

The toxic effects of hyperglycemia and its pathophysiologic derivatives such as oxidants, hyperosmolarity, or glycation products can be exerted directly on tissues or via sustained alteration in cell signaling pathways (such as changes in phospholipids or kinases) induced by the products of glucose metabolism. It is well-established now that increased oxidative stress plays a major role in the development of diabetic
complications. Further, lipid peroxidation and antioxidant enzymes in blood have been cited as markers for vascular injury/microangiopathy in DM in several studies. Therefore, in the present study, antioxidants such as erythrocyte reduced glutathione (GSH), superoxide dismutase (SOD), plasma vitamins C and E, serum total glutathione-S-transferase (GST), ceruloplasmin (CP), and protein thiols and the lipid peroxidation product namely, erythrocyte thiobarbituric acid-reactive substance (TBARS) were estimated in patients with type 2 DM (with or without complications) and healthy individuals.

Materials and Methods

This study was conducted at the Kasturba Medical College Hospital, Manipal, India from May 2002 through 2004. The study group comprised of 99 individuals coming from different parts of Udupi District, Karnataka State (south India). Moreover, it is the first study to be reported from Udupi District of Coastal Karnataka. The participants had a similar socioeconomic status following a uniform culture and food habits. There were 46 males and 53 females with the age range of 18 – 80 years. They were examined at the Outpatient section of the Medicine Department of the hospital at Manipal. Each patient, enrolled into the study, underwent a detailed routine clinical and laboratory examination including complete blood count, blood sugar values (fasting/postprandial), and glycosylated hemoglobin [American Diabetes Association (ADA) Criteria for Diabetics, 1997]. Retinopathy was confirmed by ophthalmologic examination and nephropathy by parameters of renal function test and analysis of urine samples for proteinuria. Neuropathy was detected mainly by clinical signs such as tingling, numbness, and loss of sensation. Further, due to economic constraints, only some patients could undertake nerve conduction studies.

Patients who were already diagnosed to have DM irrespective of the duration were also enrolled. Following the initial evaluation, the participants were categorized into groups, namely:

a. Controls that included healthy individuals who came to the hospital for a routine health check-up, were not diabetics, and whose blood sugar evaluation did not fulfill the ADA criteria for diabetes (Table 1). Body mass index (BMI) for 80% of these participants was in the nonobese category (<25.0 kg/m²) with 20% being in the overweight (25 – 29.9 kg/m²) grade.

b. Group 1 that included patients with DM but without complications. These patients underwent detailed evaluation for diabetic complications using Dycks criteria. Of them, 11 patients had associated essential hypertension, 15 patients were on oral hypoglycemic agents (OHA), 10 were on combination of OHA and insulin, two on insulin alone, and two on dietary regimentation alone. BMI grade for 65% of these patients was non-obese with the remaining (35%) being obese (≥30 kg/m²).

c. Group 2 consisted of patients with DM and its complications. Of these 39 patients, 17 had neuropathy alone as microvascular complications, eight patients had neuropathy with nephropathy, seven had neuropathy, nephropathy, and retinopathy, two patients had neuropathy and retinopathy, three had nephropathy and retinopathy and two had retinopathy alone. In this group 18 patients had associated hypertension, 15 were on OHA, 16 were on combination of OHA and insulin, and eight were on insulin alone. The BMI grade for 75% of these patients was overweight with 15% being obese and the remaining (10%) being nonobese.

Table 1. Demographic data and clinical characteristics of the diabetic patients and healthy controls.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Diabetics without complications (group 1)</th>
<th>Diabetics with complications (group 2)</th>
<th>Healthy subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>29</td>
<td>39</td>
<td>31</td>
</tr>
<tr>
<td>Gender (M:F)</td>
<td>12:17</td>
<td>21:18</td>
<td>15:16</td>
</tr>
<tr>
<td>Age (years)*</td>
<td>54.5 (49.5 – 64.25)</td>
<td>57 (50 – 63.5)</td>
<td>47 (36 – 57)</td>
</tr>
<tr>
<td>Duration of DM (years)*</td>
<td>3 (1.5 – 6)</td>
<td>10 (7 – 16)</td>
<td>—</td>
</tr>
<tr>
<td>Glycosylated hemoglobin (%)*</td>
<td>9.6 (7.1 – 12.55)</td>
<td>10.6 (8.4 – 13.10)</td>
<td>5.4 (5.1 – 5.8)</td>
</tr>
</tbody>
</table>

*Data represents median (IQR), DM=diabetes mellitus.
Exclusion criteria
Patients with macrovascular complications such as cardiovascular, cerebrovascular, and peripheral vascular diseases were excluded. Further, patients who presented with febrile illness, diabetic ketoacidosis, renal failure, and those who were suffering from chronic diseases were also excluded from the study.

This study was approved by the Institutional Ethics Committee. Informed consent was obtained from each participant before obtaining the blood sample.

Sample collection and preparation
Convenient samples of blood were collected into EDTA bottles from the participants on admission to the hospital when the diagnosis was confirmed and before starting the treatment. The erythrocyte suspension was prepared according to the method of Beutler et al. It was immediately centrifuged under refrigeration at 3000 g for 10 minutes. Plasma and the buffy coat were carefully removed and the separated cells were washed thrice with cold saline phosphate buffer, pH 7.4 (sodium phosphate buffer containing 0.15 mol/L NaCl).

The erythrocytes were then suspended in an equal volume of physiologic saline. Appropriately diluted hemolysates were then prepared from erythrocyte suspension by the addition of distilled water for the estimation of GSH, SOD, and TBARS. The plasma was used for the estimation of vitamins C and E.

Serum separation
Two milliliters of venous blood was collected from hospitalized patients and control groups under aseptic precautions, in a clean dry centrifuge tube. The blood samples were allowed to clot for one hour and then centrifuged at 1600 g under refrigeration for 30 minutes at 4°C. The estimations were done within two days of blood collection.

The erythrocyte GSH was estimated colorimetrically. GSH reacts with 5',5'-dithiobis(2-nitrobenzoic acid, DTNB), a disulfide chromogen which is readily reduced by it to an intensely yellow compound measured at 412 nm. Serum total GSH was analyzed spectrophotometrically. Reduced glutathione was incubated with 1-chloro-2,4-dinitrobenzene (CDNB) in the presence of serum containing GST to form 2,4-dinitrophenylglutathione (adduct), which was read at 340 nm. Serum total protein thiols were estimated by a spectrophotometric method using DTNB, which reacts with accessible SH groups in proteins and reduces to stable intermediate compound of mixed disulfide, protein-S-S-aromatic compound. The reduced product of DTNB is 5-mercaptop-2-nitrobenzoate (MNB). The yellow color developed is measured at the end of five minutes at 412 nm.

Serum CP was determined colorimetrically. At pH 5.4, CP catalyzes the oxidation of p-phenylene diamine (PPD) to yield a colored product, which is believed to correspond either to Bandrowski’s base or to Wuerter’s red. The rate of formation of colored oxidation product is proportional to the concentration of serum CP if a correction is made for nonenzymatic oxidation of PPD. Therefore, simultaneous assays are carried out with and without sodium azide, which inhibits the enzymatic oxidation of PPD. The difference between the results of the two assays is proportional to the CP concentration. The PPD-oxidase reaction is subject to a lag phase, owing to the oxidation of serum ascorbic acid. To avoid this potential source of error, timing of the reaction is delayed until after the lag phase.

Red cell SOD was analyzed by the method of Beauchamp and Fridovich. Inhibition of the reduction of nitroblue tetrazolium (NBT) by superoxide radicals, generated by the illumination of riboflavin in the presence of oxygen and an electron donor, methionine, was used as the basis for the assay of SOD.

Plasma vitamin C was determined colorimetrically using 2,4-dinitrophenyl hydrazine (DNPH) as a color compound. Ascorbic acid is oxidized by copper to form dehydroascorbic acid, which then reacts with acidic DNPH to form red bishydrazone. Absorbance at 520 nm is measured.

Plasma vitamin E was measured colorimetrically using Emmorie-Engel reaction, which is based on the reduction of iron by tocopherols from ferric to ferrous state. It then forms a red complex with α-α₁ dipyridyl.

Tocopherols and carotenoids were extracted into petroleum ether and the absorbance read at 450 nm to measure the carotenoids. A correction for the carotenoids was made after adding ferric chloride and reading at 520 nm.

Erythrocytic lipid peroxidation products were quantified by the thiobarbituric acid method. TBARS reacted with thiobarbituric acid to form a colored adduct that had a maximum absorbance at 532 nm. The hemoglobin content of the
erythrocyte was estimated by the cyanmethemoglobin method. Hemoglobin was treated with Drabkin’s reagent containing potassium ferricyanide, potassium cyanide, and potassium dihydrogen phosphate. The ferricyanide oxidized hemoglobin to methemoglobin, which was converted to cyanmethemoglobin by the cyanide. Absorbance was measured at 540 nm.21

Reagents
Special chemicals like DTNB, NBT, 2,2-dipyridyl-alpha tocopherol were obtained from Sigma-Aldrich Chemicals, USA. All the other reagents used were of analytical grade obtained from Merck India Ltd., Mumbai and Ranbaxy laboratories Ltd., Punjab.

Instruments used
Mettler weighing balance, Vortex machine, Remi low-speed centrifuge, Merck autopipettes, and Spectrophotometer-Genesys 10UV were used.

Statistical analysis
Statistical analysis was carried out by the non-parametric Kruskal-Wallis test by using the SPSS software version 11.

Results
Erythrocytic GSH was significantly lower (P<0.05) in group 1 compared with the controls. However, an apparent decrease was also observed in group 2 [95%CI (-3.43, 6.02)]. An increase in GSH was seen in group 2 compared with group 1 [95%CI (-8.05, 1.11)] (Table 2). Serum total GST was seen to be elevated in both groups 1 and 2 [95%CI (-1.66, 0.75) and (-1.84, 0.50)]. It was also higher in group 2 on an intergroup comparison [95%CI (-1.37, 0.93)].

The levels of protein thiols first rose in group 1 compared with the controls [95%CI (-111.1, 170.3)]. Thiols were lower in group 2 [95%CI (-97.91, 194.5)] in comparison with the controls [95%CI (-134.9, 172.2)]. However, these findings

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (C)</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH (mg/g Hb)</td>
<td>18.43 (14.61 – 22.62)</td>
<td>12.23 (9.06 – 18.53)</td>
<td>17.36 (12.32 – 21.48)</td>
<td>C vs. group1 P&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>n=24</td>
<td>n=24</td>
<td>n=23</td>
<td></td>
</tr>
<tr>
<td>GST (IU/L)</td>
<td>1.46 (0.63 – 2.61)</td>
<td>1.66 (1.04 – 3.75)</td>
<td>1.98 (0.83 – 3.54)</td>
<td>C vs. group1 P=0.41</td>
</tr>
<tr>
<td></td>
<td>n=25</td>
<td>n=23</td>
<td>n=34</td>
<td></td>
</tr>
<tr>
<td>Thiol (µmol??/L)</td>
<td>230.00 (145.0 – 532.50)</td>
<td>265.00 (120.0 – 382.50)</td>
<td>215.0 (170.0 – 340.0)</td>
<td>C vs. group1 P=0.79</td>
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<tr>
<td></td>
<td>n=20</td>
<td>n=14</td>
<td>n=16</td>
<td></td>
</tr>
<tr>
<td>Ceruloplasmin (mg/dL)</td>
<td>45.15 (28.56 – 77.02)</td>
<td>36.26 (25.24 – 46.16)</td>
<td>47.40 (31.88 – 61.38)</td>
<td>C vs. group1 P=0.22</td>
</tr>
<tr>
<td></td>
<td>n=26</td>
<td>n=24</td>
<td>n=36</td>
<td></td>
</tr>
<tr>
<td>Superoxide dismutase (U/g Hb)</td>
<td>885.42 (679.97 – 1167.65)</td>
<td>986.57 (698.73 – 1608.60)</td>
<td>1083.99 (663.94 – 1480.80)</td>
<td>C vs. group1 P=0.49</td>
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<tr>
<td></td>
<td>n=26</td>
<td>n=24</td>
<td>n=32</td>
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<tr>
<td>Vitamin C (µmol??/L)</td>
<td>1.27 (0.71 – 1.71)</td>
<td>1.44 (1.15 – 2.30)</td>
<td>1.42 (0.88 – 1.78)</td>
<td>C vs. group1 P=0.08</td>
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<tr>
<td></td>
<td>n=31</td>
<td>n=29</td>
<td>n=39</td>
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<tr>
<td>Vitamin E (mg/dL)</td>
<td>1.08 (0.80 – 1.55)</td>
<td>1.17 (0.95 – 1.55)</td>
<td>1.19 (0.91 – 1.51)</td>
<td>C vs. group1 P=0.64</td>
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<tr>
<td></td>
<td>n=31</td>
<td>n=27</td>
<td>n=38</td>
<td></td>
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<tr>
<td>TBARS (nM/g Hb)</td>
<td>4.89 (2.63 – 10.12)</td>
<td>3.85 (1.38 – 8.08)</td>
<td>6.70 (2.52 – 13.48)</td>
<td>C vs. group1 P=0.21</td>
</tr>
<tr>
<td></td>
<td>n=31</td>
<td>n=28</td>
<td>n=39</td>
<td></td>
</tr>
</tbody>
</table>

IQR=inter quartile range, NS=nonsignificant, n=sample size, *nonparametric Kruskal-Wallis test, TBARS=thiobarbituric acid- reactive substance.
were not statistically significant.

Serum CP levels were lower in group 1 [95%CI (-18.18, 28.11)] and elevated in group 2 [95%CI (-24.56, 20.32)] compared with the controls. Group 2 had higher levels compared with group 1 [95%CI (-29.07, 14.91)].

Erythrocyte SOD and plasma vitamins C and E showed a higher levels in both the groups 1 [95%CI (-1331, 1150), (-1.17, 0.05), and (-0.53, 0.23), respectively] and 2 [95%CI (-1782, 656.8), (-0.67, 0.37), and (-0.55, 0.20), respectively] compared with the controls. These were elevated in group 2 compared with group 1 [95%CI (-1680, 735.30), (-0.09, 0.93), and (-0.41, 0.34), respectively] (Table 2).

Erythrocytic TBARS decreased in group 1 [95%CI (-6.87, 11.82)] compared with the controls, but increased in group 2 [95%CI (-14.56, 3.73)]. An intergroup comparison showed an increased TBARS levels in group 2 [95%CI (-16.96, 1.18)]. The coefficient of variance data (CV%) is depicted in Table 3.

**Discussion**

In the present study, erythrocyte GSH was significantly reduced in DM-group 1 compared with the controls. The same trend was observed in DM with complications (group 2), although not significant. An intergroup (group 1 versus group 2) comparison showed an insignificant increase in group 2. Other researchers have also reported a similar observation of a decrease in GSH in patients with type 2 DM. Serum total GST, which uses GSH as one of its substrates was found to be higher in both groups 1 and 2 compared with the controls; although, the results were not significant. The GST levels were higher in group 2 compared with group 1. An increase in GST has also been reported in streptozotocin-induced diabetic rat tissues, earlier.

Serum protein thiol levels showed an increase in the diabetic group 1 and a decrease in group 2 compared with the controls. When an intergroup (group 1 versus group 2) comparison was made, a decrease was observed in group 2, although insignificant. There have been reports on decreased plasma thiol levels in diabetic patients recently.

Therefore, decreased red blood cell GSH and serum protein thiols as well as increased serum total GST levels may be due to a compensatory mechanism of the antioxidants to combat the oxidative stress in diabetic conditions with or without complications.

Ceruloplasmin is a free radical scavenger as well as a late acute phase reactant protein. It is involved in iron metabolism. Further, it has also been suggested that metals, especially copper, might be increased in the diabetic state. Thus, it influences the availability of the iron in free radical generating reactions. Considering the pro-oxidant status of patients with DM, lower CP levels in group 1 may be due to the adaptive response of CP as an antioxidant. Further, an increase in the level of CP in group 2 probably favors its protective action against free radical injury. These changes are however insignificant in this study, but the tendency of serum CP to increase in type 2 DM has also been reported in a recent study.

The levels of erythrocytic antioxidant enzyme SOD also showed an apparent increase in both groups 1 and 2 compared with the controls probably to counteract the free radical generation in the diabetic state. Earlier reports on SOD levels in DM however indicate a decrease in the levels and are contradictory to our findings. An intergroup comparison also showed an increase in the SOD in group 2 in this study.

The plasma antioxidant vitamins C and E showed increased levels in groups 1 and 2 compared with the controls. However, the levels of vitamin C and E were found to be almost the same when an intergroup comparison was made. Vitamin C lowers sorbitol level, which is harmful.

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**Table 3. Coefficient of variance (CV%) values for all the parameters in the three groups.**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>DM-without complications</th>
<th>DM-with complications</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>30.364</td>
<td>104.786</td>
<td>77.890</td>
</tr>
<tr>
<td>Vit C</td>
<td>53.053</td>
<td>68.632</td>
<td>47.351</td>
</tr>
<tr>
<td>CER/CP</td>
<td>56.103</td>
<td>78.012</td>
<td>74.380</td>
</tr>
<tr>
<td>TBARS</td>
<td>106.626</td>
<td>138.804</td>
<td>183.600</td>
</tr>
<tr>
<td>GST</td>
<td>82.762</td>
<td>86.629</td>
<td>81.287</td>
</tr>
<tr>
<td>SOD</td>
<td>59.331</td>
<td>194.457</td>
<td>54.771</td>
</tr>
<tr>
<td>Vit E</td>
<td>45.468</td>
<td>54.558</td>
<td>50.698</td>
</tr>
<tr>
<td>Thiol</td>
<td>65.411</td>
<td>62.335</td>
<td>57.000</td>
</tr>
</tbody>
</table>

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to the eyes and kidneys in patients with DM. Further, it decreases protein loss through the urine and improves glucose tolerance in type 2 DM. Studies on the effect of vitamin E supplementation and deprivation in DM have been carried out in humans as well as on experimental animals.\textsuperscript{5,30–32} Supplementation protects free radical damage in diabetic state. However, in the present study, care has been taken to keep the nutritional differences between the groups to a minimum.

Peroxidative damage as assessed by erythrocyte TBARS levels in this study showed a decrease in group 1 but an elevation in group 2 compared with the controls. Within groups, TBARS levels were higher in group 2. This indicates resistance to oxidative damage in group 1, probably because of the protective antioxidant mechanisms quoted above. However, it fails with the severity of the disease. There are many studies on the increased oxidative damage manifested as TBARS in DM.\textsuperscript{2,5} Further, increased total antioxidant power with normal lipid peroxidation in plasma and saliva of patients with type 1 DM has been reported earlier.\textsuperscript{33} In another study, the effect of pentoxifylline has been evaluated in patients with type 2 DM where it was noted that there was a decrease in lipid peroxidation in the plasma with no effect on the total antioxidant power.\textsuperscript{34}

Thus, it can be concluded that in the present study a significant decrease in erythrocyte GSH as well as increase or decrease of other antioxidants reflects the overwhelming adaptive response to the challenge of oxidative stress in the diabetic state with or without complications. This response might be helpful in reducing free radical induced oxidant injury. A fact that needs mention at this juncture is that the diabetic state is a paradox in itself. Williamson et al. noted an increased cellular reduced nicotinamide adenine dinucleotide/oxidized nicotinamide adenine dinucleotide (NADH/NAD) ratio and proposed that diabetes was a state of “reductive” stress and “pseudohypoxia”.\textsuperscript{35} They raised the question of how oxidative damage might arise in a reducing environment. Moreover, the popular notion of generalized oxidant stress in DM lost support because of the lack of evidence.

Baynes and Thorpe proposed a greater role for overload of metabolic pathways as the primary culprit in oxidant stress in this condition.\textsuperscript{36} Further, this study besides its limitations and observing the trend in levels of various parameters (refer 95%CI in results) supports the observation that appropriate doses of antioxidants especially antioxidant vitamins can be prescribed to patients with DM and its complications to prevent any oxidative damage.\textsuperscript{37} Besides, transition metal-chelating agents and hydroxyl radical scavengers may prove useful as adjuvants to other forms of treatment. However, a study carried out on a larger sample size may be more decisive. Because our center was a tertiary hospital, we could not be very selective about the patients—a limitation of this study. Moreover, the patients in this study were already on either monotherapy or combination of antidiabetic therapy. It is well known that most of the OHAs also have potent antioxidant effects.\textsuperscript{38,39} Hence, it could be one of the reasons why most of the parameters of this study do not differ in the diabetic patients compared with the controls, again a limitation of this study.

References

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