TO EVALUATE THE ANTIOXIDANT STATUS IN TYPE 2 DIABETIC PATIENTS WITH OR WITHOUT NEPHROPATHY

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ABSTRACT
Oxidative stress is a condition in which the cellular production of reactive oxygen species (ROS) exceeds the physiological capacity of the antioxidant defense system to render ROS inactivate. Objective: To estimate the levels of Vitamin C and GSH in type 2 diabetic patients. To find out the correlation, if any, between glycemic control and the levels of Vitamin C and GSH. Method: The study was conducted on patients attending rural medical college hospital of India. The sample was analysed for estimation of fasting blood glucose, glycated Hb, Vitamin C and GSH. Statistical analysis: Data obtained were analyzed as per standard statistical methods. Results: Out of 150 subjects, Fasting blood glucose (FBG) and glycated hemoglobin was higher in diabetic patients with microalbuminuria than in those without microalbuminuria. Highly significant increase in FBG and HbA1c in non-complicated and complicated cases as compared to healthy controls. Vitamin C level and GSH were significantly decreased in non-complicated and complicated cases. Conclusion: Antioxidant levels decrease in diabetic patients, more so with complications. Combinations of antioxidants given as dietary supplements seem to offer the most promising for achieving clinical breakthroughs.

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INTRODUCTION

One adult in ten will have diabetes by 2030; figures signify that the number of people living with diabetes is estimated to rise from 366 million in 2011 to 552 million by 2030, if no urgent action is taken. This equates to roughly three new cases every ten seconds or almost ten million per year, between 2010 and 2030, there will be a 69% increase in number of adults with diabetes in developing countries and a 20% increase in developed countries [1]. Insulin resistance and abnormal insulin secretion are central to the development of type 2 diabetes mellitus (T2DM). Various theories explain the pathophysiology of T2DM. Among them, one theory is based on the observation that hyperglycemia increases glucose metabolism via the sorbital pathway. Intracellular glucose is predominantly metabolised by phosphorylation and subsequent glycolysis, but when increased, some glucose is converted to sorbitol by the enzyme aldolase reductase. Increased sorbital concentration alters redox potential, increases cellular osmolality, generates reactive oxygen species (ROS), and likely leads to the other types of cellular dysfunction.

However, testing of this theory in humans, using aldose reductase inhibitors, has not demonstrated significant beneficial effects on clinical endpoints of retinopathy, neuropathy, or nephropathy. A possible unifying mechanism is that hyperglycemia leads to increased production of ROS or superoxide in the mitochondria; these compounds may activate all four of the pathways: formation of AGEs, sorbitol pathway, activation of PKC pathway, and hexosamine pathway [2]. The free radicals are involved in various pathophysiological disorders in humans such as atherosclerosis, cancer, arthritis, gastritis and diabetes. Free radicals contains unpaired electrons which are produced as adducts produced by normal metabolic reactions and can readily react with cellular biomolecules like DNA, proteins and lipids. Antioxidants are capable of protecting the normal cells from the highly reactive free radicals by donating a pair of electron and make them non-reactive [3] The balance between the rate of free radical (FR) generation and elimination is important. Excess cellular radical generation can be harmful. However, if there is a significant increase in radical generation, or a decrease in radical elimination from the cell, oxidative cellular stress ensues. There is convincing experimental and clinical evidence that the generation of ROS increases in both types of diabetes and that the onset of diabetes is closely associated with oxidative stress.[4] Oxidative stress results from increased ROS and/ or RNS. The possible sources of oxidative stress in diabetes might include auto-oxidation of glucose, shifts in redox balances, decreased tissue concentrations of low molecular weight antioxidants, such as reduced glutathione (GSH) and vitamin E, and impaired activities of antioxidant defence enzymes such as SOD and CAT. [5] ROS generated by high glucose is causally linked to elevated glucose and other metabolic abnormalities important to the development of diabetic complications. However, the exact mechanism by which oxidative stress may contribute to the development of diabetic complications is undetermined. [6] The underlying causes of oxidative stress in diabetes may include, high glucose concentrations which itself acts as a pro-oxidant, production of glycated proteins and glycation of antioxidative enzymes, which limit their capacity to detoxify oxygen radicals. [7] Stimulate cytochrome p450-like activity by excessive NADPH produced by glucose metabolism.

Diabetes overloads glucose metabolic pathways, resulting in excess FR production and oxidative stress.[8] Implication of oxidative stress in the pathogenesis of diabetes is suggested, not only by oxygen free-radical generation, but also due to non enzymatic protein glycosylation, auto-oxidation of glucose,[9] impaired glutathione metabolism,[10] alteration in antioxidant enzymes, lipid peroxides formation and decreased ascorbic acid levels.[11] As the disease progresses, antioxidant potential decreases, and the plasma lipid peroxidation products increase depending upon the level of glycemic control.

In diabetic patients an imbalance between ROS production and antioxidant levels has been reported but there is still lack of data regarding the actual status of antioxidants in T2DM patients. Thus, this study was designed to report the levels of antioxidant vitamin C and erythrocytic GSH in T2DM yet to be treated.

AIMS AND OBJECTIVES:

To estimate the levels of Vitamin C and erythrocytic GSH in T2DM patients. To find out the correlation, if any, between glycemic control and the levels of Vitamin C and GSH. The study was done to evaluate the antioxidant status in T2DM patients with and without nephropathy. Diabetes with Nephropathy (microalbuminuria) was taken as the complicated cases.

MATERIALS AND METHODS:

The present study was conducted in the Department Of Biochemistry, in collaboration with Department of Medicine, MMIMSR (Maharishi Markandeshwar Institute of Medical Science and Reasearch), Mullana (Ambala), India. The sources of the subjects were from Medicine OPD/IPD of the MM Teaching Hospital. Total of 150 subjects were taken for the study which were divided into three groups.

GROUP 1: 50 Patients with T2DM, without nephropathy.
GROUP 2: 50 Patients with T2DM with nephropathy.
CONTROLS: 50 Age and sex matched healthy volunteers were taken.
All the subjects were in the age group of 30-70 years.

INCLUSION CRITERIA:
Patients were included in the study if: 1) FBG≥126mg/dl. 2) Random blood glucose≥200mg/dl, 3) Glycated haemoglobin≥48mmol/mol (or ≥6.5%) (ADA). [12]
Criteria for diabetic nephropathy- Albumin to creatinine ratio (ACR). ACR ≥ 30 μg/mg -Microalbuminuria [13]
EXCLUSION CRITERIA: Following patients were excluded from the study: 1) Patients with macrovascular complications such as cardiovascular, cerebrovascular, and peripheral vascular diseases. 2) Patients with febrile illness, diabetic ketoacidosis, renal failure, and those who were suffering from chronic diseases.

SAMPLE COLLECTION:
Analysis in Serum:
5ml of venous blood was collected into EDTA vials from each subject aseptically from the anticubital vein after 12 hours of overnight fast. The sample was analysed the same day for estimation of fasting blood glucose (FBG), glycated Hb (GHB), Vitamin C and GSH.
- FBG- by Glucose oxidase-peroxidase (GOD-POD) method. [14]
- GHb- by Ion Exchange Resin method. [15]
- Plasma Vitamin C-Colorimetric method (Beutler E).[16]
- GSH-Colorimetric method (Omaye ST).[17]

Analysis in Urine:
Random urine sample was collected in sterile container for determination of urinary albumin and creatinine.
- Albumin: Estimation was done by pyrogallol red method.[18]
- Creatinine: Estimation was done by Jaffe’s alkaline picrate method.[19]

Albumin creatinine Ratio was determined.
ACR = Concentration of albumin/concentration of creatinine=µg albumin/mg creatinine
ACR ≥ 30 µg/mg -Microalbuminuria.

ETHICAL CONSIDERATIONS
The study was approved by the Ethics Committee of Maharishi Markandeshwar Institute of Medical Sciences and Research. The approval was on the agreement that patient anonymity must be maintained, good laboratory practice, quality control ensured, and that every finding would be treated with utmost confidentiality and for the purpose of this research only. All work was performed according to the International Guidelines for Human Experimentation in Biomedical Research [20]. Approval was obtained from the subjects by taking the informed consent.

OBSERVATION
The present study was conducted in Department of Biochemistry in collaboration with Department of Medicine Maharishi Markandeshwar Institute of Medical Science and Reasearch (MMIMSR), Mullana, Ambala, Haryana (INDIA). 50 Type-2 diabetics with nephropathy as a complication, 50 Type-2 diabetics without complication and 50 age matched healthy controls were enrolled as subjects in the study.

The sex-wise distribution of the subjects showed that out of 50 non-complicated cases 28 were males and 22 were females. In 50 complicated cases, 22 were males and 28 were females and in out of 50 healthy controls 28 were males and 22 were females.

![Figure 1: Showing mean Fasting blood glucose in healthy controls, non-complicated and complicated diabetic cases.](image-url)
Figure 2: Showing mean HbA1c levels in healthy controls, non-complicated and complicated diabetic cases.

Figure 3: Showing mean Vitamin C levels in healthy controls, non-complicated and complicated diabetic cases.

Figure 4: Showing mean Reduced glutathione levels in healthy controls, non-complicated and complicated diabetic cases.
Table 1: Mean value, standard deviation and p value of FBG, HbA1c, Vitamin C, and GSH between healthy control and non-complicated cases.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean±SD</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBG Controls</td>
<td>50</td>
<td>84.04±10.060</td>
<td>0.000</td>
</tr>
<tr>
<td>Non-complicated</td>
<td>50</td>
<td>163.59±76.905</td>
<td>0.000</td>
</tr>
<tr>
<td>HbA1c Controls</td>
<td>50</td>
<td>6.76±0.703</td>
<td>0.000</td>
</tr>
<tr>
<td>Non-complicated</td>
<td>50</td>
<td>10.30±2.591</td>
<td>0.000</td>
</tr>
<tr>
<td>Vitamin C Controls</td>
<td>50</td>
<td>125.70±13.45</td>
<td>0.002</td>
</tr>
<tr>
<td>Non-complicated</td>
<td>50</td>
<td>97.22±78.39</td>
<td>0.000</td>
</tr>
<tr>
<td>GSH Controls</td>
<td>50</td>
<td>165.90±68.915</td>
<td>0.480</td>
</tr>
<tr>
<td>Non-complicated</td>
<td>50</td>
<td>154±98.943</td>
<td>0.480</td>
</tr>
</tbody>
</table>

In healthy control, mean value of FBG and HbA1c was 84.04±10.060 mg/dl (p<0.001) and 6.76±0.703% (p<0.001) respectively. The mean Vitamin C and GSH level was 125.70±13.45 mg/gHb (p<0.05) and 165.90±68.915 µmol/gHb (p>0.05). In non-complicated cases, mean value of FBG was 163.59±76.905mg/dl (p<0.001), mean HbA1c was 10.30±2.591% (p<0.001), mean Vitamin C was 97.22±78.39mg/gHb (p<0.05) and mean of GSH was 154.61±98.943 µmol/gHb (p>0.05).

Table 2 Mean values, standard deviation and p value of FBG, HbA1c, Vitamin C, and GSH between healthy control and complicated cases.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean±SD</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBG Controls</td>
<td>50</td>
<td>84.04±10.060</td>
<td>0.000</td>
</tr>
<tr>
<td>Complicated</td>
<td>50</td>
<td>194.02±105.238</td>
<td>0.000</td>
</tr>
<tr>
<td>HbA1c Controls</td>
<td>50</td>
<td>6.76±0.703</td>
<td>0.000</td>
</tr>
<tr>
<td>Complicated</td>
<td>50</td>
<td>10.68±2.831</td>
<td>0.000</td>
</tr>
<tr>
<td>Vitamin C Controls</td>
<td>50</td>
<td>125.70±13.45</td>
<td>0.000</td>
</tr>
<tr>
<td>Complicated</td>
<td>50</td>
<td>38.13±8.206</td>
<td>0.000</td>
</tr>
<tr>
<td>GSH Controls</td>
<td>50</td>
<td>165.90±68.915</td>
<td>0.031</td>
</tr>
<tr>
<td>Complicated</td>
<td>50</td>
<td>131.16±67.104</td>
<td>0.031</td>
</tr>
</tbody>
</table>

In healthy control, mean value of FBG and HbA1c was 84.04±10.060 mg/dl (p<0.001) and 6.76±0.703% (p<0.001) respectively. The mean Vitamin C and GSH level was 125.70±13.45 mg/gHb (p<0.05) and 165.90±68.915 µmol/gHb (p>0.05). In Complicated cases, mean value of FBG was 194.02±105.238mg/dl (p<0.001), mean HbA1c was 10.68±2.831% (p<0.001), mean Vitamin C was 38.13±8.206mg/gHb (p<0.001) and mean of GSH was 131.16±67.104 µmol/gHb (p<0.05).

Table 3 Pearson correlations (r) between the parameters of non-complicated cases.

<table>
<thead>
<tr>
<th>Age</th>
<th>N</th>
<th>FBG</th>
<th>HbA1c</th>
<th>Vitamin C</th>
<th>GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pearson correlation</td>
<td>1</td>
<td>0.036</td>
<td>-0.082</td>
<td>-0.130</td>
<td>0.062</td>
</tr>
<tr>
<td>Sign(2-tailed)</td>
<td>50</td>
<td>0.801</td>
<td>0.572</td>
<td>0.369</td>
<td>0.670</td>
</tr>
<tr>
<td>FBG</td>
<td>Pearson correlation</td>
<td>0.036</td>
<td>1</td>
<td>0.373</td>
<td>-0.207</td>
</tr>
<tr>
<td>Sign(2-tailed)</td>
<td>50</td>
<td>0.801</td>
<td>0.008</td>
<td>0.148</td>
<td>0.255</td>
</tr>
<tr>
<td>HbA1c</td>
<td>Pearson correlation</td>
<td>-0.082</td>
<td>0.373</td>
<td>1</td>
<td>-0.171</td>
</tr>
<tr>
<td>Sign(2-tailed)</td>
<td>50</td>
<td>0.572</td>
<td>0.008</td>
<td>0.235</td>
<td>0.163</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>Pearson correlation</td>
<td>-0.130</td>
<td>-0.207</td>
<td>-0.171</td>
<td>1</td>
</tr>
<tr>
<td>Sign(2-tailed)</td>
<td>50</td>
<td>0.369</td>
<td>0.148</td>
<td>0.235</td>
<td>0.877</td>
</tr>
<tr>
<td>GSH</td>
<td>Pearson correlation</td>
<td>0.062</td>
<td>0.164</td>
<td>0.200</td>
<td>0.022</td>
</tr>
<tr>
<td>Sign(2-tailed)</td>
<td>50</td>
<td>0.670</td>
<td>0.255</td>
<td>0.163</td>
<td>0.877</td>
</tr>
<tr>
<td>N</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

*.Correlation is significant at the p value < 0.05 level (2-tailed).
Table 3 shows the correlation between the parameters of non-complicated cases where we found there was a negative correlation between age and HbA\(_{1c}\), age and vitamin C, FBG and vitamin C and HbA\(_{1c}\) and vitamin C with r value -0.082 (p>0.05), -0.130 (p>0.05), -0.207 (p>0.05) and -0.171 (p>0.05) respectively.

Table 4 shows the correlation between the parameters of complicated diabetic type II group. Vitamin C is increasing with increasing age, FBG and HbA\(_{1c}\). The r value between vitamin C and age was 0.026 (p>0.05) which was not satisfactory significant. Similarly, r value between vitamin C and FBG was 0.249 (p>0.05) and r between vitamin C and HbA\(_{1c}\) was 0.095 (p>0.05) which was not satisfactory significant.

GSH in complicated diabetes was also increasing age and HbA\(_{1c}\). The r value between GSH and age was 0.015 (p>0.05) and r between GSH and HbA\(_{1c}\) was 0.181 (p>0.05).
Figure 6: Showing correlation between FBG and Reduced glutathione in complicated diabetic cases.

GSH in complicated diabetes was also increasing age and HbA1c. The r value between GSH and age was 0.015 (p>0.05) and r between GSH and HbA1c was 0.181 (p>0.05).

DISCUSSION

DM is associated with endothelial dysfunction and oxidative stress. Chronic exposure to elevated glucose and fatty acid concentrations can cause damage in different types of cells by a variety of mechanisms collectively known as glucolipotoxicity, and oxidative stress may be a common link. The oxidative stress in DM is greatly increased due to prolonged exposure to glycemia and impairment of the oxidant/antioxidant balance. Lipids are among the primary targets of oxidative stress. Lipid peroxidation of the cellular structures, a consequence of increased OFRs, is thought to play an important role in atherosclerosis and microvascular complications of T2DM [21]

Mean FBG in control, non-complicated and complicated cases was 84.04 mg/dl, 163.59 mg/dl and 194.02 mg/dl respectively. Highly significant increase in FBG level. FBG was highly significant in non-complicated (p<0.001) and in complicated (p<0.001) cases as compared to healthy controls.

Khan MI et al. found that fasting and post-prandial blood glucose concentration were significantly higher (p<0.0001) in patients with microalbuminuria than in those without microalbuminuria. Similarly, HbA1c was significantly higher in diabetic patients with microalbuminuria in comparison to without microalbuminuria.[22] Marked increase in HbA1c level was found in diabetic patients with microalbuminuria as compared to without microalbuminuria could be attributed to uncontrolled/ persistent higher blood sugar level as indicated by excessive glycosylation of hemoglobin. Piarulli et al. also supported his study. Mean HbA1c level of healthy control, non-complicated and complicated cases was 6.76%, 10.30% and 10.68% respectively. There was a significant increase in HbA1c level. HbA1c was highly significant in non-complicated (p<0.001) and in complicated (p<0.001) cases as compared to healthy controls.

Murugan K et al. also showed in their study that diabetes associated nephropathy (DM+NP) showed significant elevated levels of HbA1c in blood. From investigation he observed that there was a strong relationship between fasting blood sugar level, post-prandial blood sugar level and HbA1c level in diabetic patients.[24] Black GJ reported that the blood glucose and HbA1c levels considerably increase in diabetic patients.[25] Bernadette B.A. and other researchers reported elevated levels of HbA1c in diabetes.[26] In the present study, also reported that the FBG and HbA1c increase in diabetic patients.

Mean Vitamin C level of healthy control, non-complicated and complicated cases was 125.70 mg/dl, 97.22mg/dl and 38.13mg/dl respectively. There was a significant decrease in Vitamin C level in non-complicated and complicated cases as compared to controls. Vitamin C was significant in non-complicated (p<0.05) cases and highly significant in complicated cases (p<0.001) as compared to healthy controls.

Srivatsan R et al. showed that the plasma antioxidant vitamin C showed apparent decrease in both groups 1 and 2 compared with the controls. An intergroup (group 1 versus group 2) comparison showed an insignificant increase in group 1. Vitamin C lowers sorbitol level, which is harmful to the eyes and kidneys in patients with DM. Further, it decreases protein loss through the urine and improves glucose tolerance in T2DM. [27]

Studies by Som et al. and Stankova L et al. have reported lowered plasma concentrations of ascorbic acid in diabetics compared to healthy subjects. [28, 29] The presented study by Hisalkar PJ et al. also supports that low vitamin C status in diabetes may be due to a higher turnover rate of ascorbic acid, with increased oxidation to the oxidized form dehydroascorbate.[30] Takahishi N et al. demonstrated that renal dysfunction was associated with a continuous decrease in plasma vitamin C concentration in both diabetic and non-diabetic patients with CKD. Moreover, vitamin C concentration was significantly lower at any given eGFR in diabetic patients compared with non-diabetic patients.[31]
The present study also showed that vitamin C significantly decrease in group 1 and group 2 with the controls but intergroup group 1 and group 2 comparison showed an significant increase in group 1.

A likely explanation for a lower Vitamin C status in diabetics is that ascorbic acid is actively transported into the cells in its partially oxidized form as dehydroascorbic acid, which is promptly converted to ascorbic acid within the cell. In study by Vivian Samuel T vitamin C showed an inverse correlation with the both glycaemic control and the duration of diabetes. [21] Similar findings were reported by Sundaram et al. in their study on antioxidant in diabetes. The carrier of ascorbic acid transport serves also to transport glucose and is inhibited in transporting ascorbic acid by the hyperglycemia of diabetics. Lysy et al. showed that in diabetics, plasma ascorbic-acid levels were negatively correlated with Glycated hemoglobin, a measure of glycemic control. [32]

The presented study showed that Vitamin C negatively correlated with age, FBG and Glycated hemoglobin in non-complicated cases and negatively correlated with GSH in healthy controls. Mean GSH level of healthy control, non-complicated and complicated cases was 165.90µmol/ gHb, 154.61 µmol/ gHb and 131.16 µmol/ gHb respectively. There was a significant decrease in GSH level in non-complicated and complicated cases as compared to controls. GSH level non-significant in non-complicated (p>0.05) cases but significant in complicated cases (p<0.05) as compared to healthy controls.

In the study by Srivatsan R et al. 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. [27] Pasaoglu H et al. have also reported a similar observation of a decrease in GSH in patients with T2DM. [33]. In the study by Vivian ST showed a decrease in GSH in diabetes and negative correlation between duration and glycemic control. Sushil K. Jain and Robert McVie showed that erythrocytes of diabetic patients have a significantly lower glutathione level compared with those of age-matched normal subjects. [21]

CONCLUSION
Oxidative stress is convincingly a key pathogenetic factor for diabetic complications and leads to a decrease in anti-oxidant levels. However, despite this strong evidence, the usefulness of antioxidants in preventing such complications is still elusive. New “antioxidants” are emerging, based on new findings on oxidative stress, in particular on how the oxidative stress is produced by the balance between FR production and antioxidant defenses. The “new antioxidant” approach includes the possibility of controlling FR production and of increasing intracellular antioxidant defenses, a concept different from the old one, when antioxidant action meant just scavenging the FRs already produced. The new view, of course, needs to be proven in clinical trials, but it seems very promising.

COMPETING INTERESTS
The author declares that there were no competing interests associated with this study

SOURCE OF SUPPORT
Nil.

REFERENCES

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