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Antioxidant and oxidative stress status in type 2 diabetes and diabetic foot ulcer

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Objective. Oxidative stress (OS) has been implicated in the aetiology and progression of diabetic complications including diabetic foot ulcer. In this study, the levels of lipid peroxides (LPO) and 8-hydroxy-2’-deoxyguanosine (8-OHdG) as well as the enzymatic antioxidant activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in type 2 diabetes mellitus and diabetic foot ulcers subjects were assessed and compared with apparently healthy normal subjects to understand the involvement of OS in the subjects.

Method. The abovementioned OS markers were measured in 50 subjects for each of the following groups: type 2 diabetes mellitus (DM), diabetic foot ulcer (DF) and non-diabetic control (NC).

Results. Significant elevated values of LPO (39.86%) and 8-OHdG (45.53%) were found in DM subjects compared with the NC subjects. This increase in both parameters was greater for DF subjects: 80.23% and 53.91% respectively. SOD activities were significantly reduced in DM (14.82%) and DF (4.09%) subjects in contrast with elevated activities of GPx observed in DM (21.87%) and DF (20.94%) subjects. Glycated haemoglobin/fasting plasma glucose (HbA1c/FPG) correlated positively with LPO, 8-OHdG and GPx, whereas a negative correlation was observed for SOD.

Conclusion. Increased oxidation subsequent to diabetic conditions induces an over-expression of GPx activity suggesting a compensatory mechanism by the body to prevent further tissue damage in the subjects.

A body of evidence exists concerning the involvement of oxidative stress (OS) in the aetiology of diabetes and its later complications, of which diabetic foot ulcer is one.1 OS arises in cells and tissues through the increased production of reactive oxygen species (ROS) and/or from decreases in the antioxidant defence system.2 Several mechanisms seem to be involved in the generation of OS in the presence of elevated glucose concentrations; they include glucose auto-oxidation, enhanced glucose flux through the polyol pathway, and non-enzymatic and progressive glycation of proteins with consequent increased formation of glucose-derived advanced glycosylation end products (AGEs).3

Under normal physiological conditions, a widespread antioxidant defence system protects the body against the adverse effects of ROS generation. The defence mechanism’s efficiency is altered in diabetes and the ineffective scavenging of free radicals may therefore play a crucial role in determining tissue damage in these subjects.4

The present investigation was carried out to assess the levels of lipid peroxides (LPO) (a marker of lipid peroxidation) and 8-hydroxy-2’-deoxyguanosine (8-OHdG) (a marker of DNA damage), as well as the enzymatic antioxidant activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in normal control (NC) type 2 diabetes mellitus (DM) and diabetic foot ulcer (DF) subjects, to understand the involvement of OS in diabetic foot ulcers.
Materials and methods

Patients
The study population comprised 50 type 2 DM subjects and 50 DF subjects with Wagner’s grade II ulcer classification (i.e. ulcer without abscess or osteomyelitis). Males and non-pregnant/non-lactating females between the ages of 40 and 60 years with HbA1c >6.5% were recruited from the medical ward of University College Hospital, Ibadan, Nigeria, as ‘test’ groups. In addition, 50 age-matched healthy non-diabetic subjects of both genders with HbA1c <6.5% were selected as a normal control group from among the staff of the same university. Informed consent was sought and obtained from each subject before recruitment into this study, which was approved by the Ethical Committee of the University of Ibadan and the University College Hospital Institutional Review Committee (UI/UCH IRC) (approval number UI/IRC/03/0096).

Blood sampling
Ten-millilitre aliquots of venous blood drawn after a 10-hour overnight fast were collected in heparinised EDTA or fluoride sample tubes and centrifuged at 3,000 rpm for 10 minutes. Plasma and haemolysate were stored at –80ºC until the day of analysis. Antioxidant enzymes (SOD, GPx) were measured in heparinised whole blood, whereas oxidative status parameters (LPO, 8-OHdG) were assessed in EDTA plasma.

Fasting plasma glucose (FPG) and glycated haemoglobin A1c (HbA1c) were measured in plasma from sodium fluoride and EDTA samples respectively, on the day of collection using standard laboratory techniques.

Analyses
Chemical reagents of the highest quality were purchased from Sigma-Aldrich, Germany. LPO concentrations were measured spectrophotometrically at 560 nm using the ferrous oxidation with xylene orange (FOX VERSION II) assay according to the method of Nourooz-Zadeh et al. This method is based on the principle of rapid peroxide-mediated oxidation of Fe²⁺ to Fe³⁺ under acidic conditions.

Plasma levels of 8-OHdG were measured at 450 nm on a microplate reader using a commercial kit from Randox Laboratories, UK. The method is based on a competitive in vitro enzyme-linked immunosorbent assay for quantitative measurement of this DNA metabolite in tissue, serum and plasma. Erythrocyte SOD activity was determined by the method of Arthur and Boyn, using a commercial kit obtained from Randox Laboratories, UK. This method uses xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitropheno)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. SOD activity is then measured by the degree of inhibition of this reaction spectrophotometrically at 505 nm.

The determination of erythrocyte GPx activity was based on modification of the method of Paglia and Valentine, using a commercial kit obtained from Randox Laboratories, UK. This method involves the oxidation of glutathione (GSH) by cumene hydroperoxide catalysed by GPx. In the presence of glutathione reductase (GR) and NADPH, the oxidised glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP⁺. The decrease in absorbance is then measured spectrophotometrically at 340 nm.

Statistical analysis
All data were presented as mean ± standard deviation (SD) for 50 subjects in each group. The Statistical Package for Social Sciences (SPSS) (version 13) was used for the statistical analysis of the data. Correlation between Pearson correlation test parameters was determined using the Statistical Package for Social Sciences (SPSS) (version 13). The correlation analysis of FPG and HbA1c with oxidant and antioxidant enzymes within NC, DM and DF groups is presented in Table I.

Table I. Correlation analysis of FPG and HbA1c with oxidant and antioxidant enzymes within NC, DM and DF groups

<table>
<thead>
<tr>
<th>Correlation between test parameters</th>
<th>Pearson correlation coefficient (p&lt;0.05)</th>
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</thead>
<tbody>
<tr>
<td>NC FPG NC LPO</td>
<td>0.919</td>
</tr>
<tr>
<td>NC FPG NC 8-OHdG</td>
<td>0.977</td>
</tr>
<tr>
<td>NC FPG NC SOD</td>
<td>–0.979</td>
</tr>
<tr>
<td>NC FPG NC GPx</td>
<td>0.979</td>
</tr>
<tr>
<td>NC FPG NC HbA1c</td>
<td>0.900</td>
</tr>
<tr>
<td>DM FPG DM LPO</td>
<td>0.985</td>
</tr>
<tr>
<td>DM FPG DM 8-OHdG</td>
<td>0.982</td>
</tr>
<tr>
<td>DM FPG DM SOD</td>
<td>–0.915</td>
</tr>
<tr>
<td>DM FPG DM GPx</td>
<td>0.926</td>
</tr>
<tr>
<td>DM FPG DM HbA1c</td>
<td>0.982</td>
</tr>
<tr>
<td>DF FPG DF LPO</td>
<td>0.842</td>
</tr>
<tr>
<td>DF FPG DF 8-OHdG</td>
<td>0.928</td>
</tr>
<tr>
<td>DF FPG DF SOD</td>
<td>–0.949</td>
</tr>
<tr>
<td>DF FPG DF GPx</td>
<td>0.823</td>
</tr>
<tr>
<td>DF FPG DF HbA1c</td>
<td>0.901</td>
</tr>
<tr>
<td>NC HbA1c NC LPO</td>
<td>0.967</td>
</tr>
<tr>
<td>NC HbA1c NC 8-OHdG</td>
<td>0.919</td>
</tr>
<tr>
<td>NC HbA1c NC SOD</td>
<td>–0.710</td>
</tr>
<tr>
<td>NC HbA1c NC GPx</td>
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<td>DM HbA1c DM LPO</td>
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<tr>
<td>DM HbA1c DM 8-OHdG</td>
<td>0.975</td>
</tr>
<tr>
<td>DM HbA1c DM SOD</td>
<td>–0.915</td>
</tr>
<tr>
<td>DM HbA1c DM GPx</td>
<td>0.906</td>
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<td>DF HbA1c DF LPO</td>
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<td>–0.849</td>
</tr>
<tr>
<td>DF HbA1c DF GPx</td>
<td>0.823</td>
</tr>
</tbody>
</table>
was used for statistical evaluation. Significance of differences was determined using one-way analysis of variance (ANOVA). Pearson’s correlation coefficient was determined within groups. Statistical significance was set at p-values <0.05.

Results

Pearson’s correlation analysis of all the groups revealed a strong correlation of FPG and HbA1c with LPO, 8-OHdG and GPx, in contrast to a negative correlation with SOD. As expected, NC-HbA1c correlated positively with NC-FPG (r=0.899). This correlation became stronger in the DM and DF groups, with a correlation coefficient of 0.982 and 0.901 respectively, as shown in Table I (p<0.05). The negative correlation between FPG and SOD (r=−0.879) in the NC group is shown in Fig. 1.

The mean values of FPG and HbA1c were respectively 214.84±65.26 mg/dl and 7.97±2.55% for the DM group. For the DF group, they were 226.93±127.10 mg/dl and 8.40±1.91% respectively, compared with 91.69±9.56 mg/dl and 4.08±0.75% for the NC group, as shown in Fig. 2a.

A high level of LPO was observed in the plasma of the DF group (56.61±17.34 μM), compared with the DM group (43.93±16.46 μM) and NC group (31.41±15.95 μM). The extent of oxidation was reflected in the concentration of DNA adduct, 8-OHdG: 49.05±13.79 μM and 46.38±18.03 μM, corresponding to an increase of 53.91% and 45.53% respectively for the DF and DM groups, compared with the NC group (31.87±11.58 μM) (Fig. 2b).

Fig. 1. Relationship between FPG and SOD in NC group. Coefficient of determination (R²): −0.773; correlation coefficient (r): −0.879 (p<0.05).

Fig. 2a. Fasting plasma glucose (FPG) and glycated haemoglobin A1c (HbA1c) in type 2 DM and DF groups, compared with the NC group. Values are statistically significant at *p<0.01. The type 2 DM and DF groups were compared with the non-diabetic NC group. The percentages of actual values of each parameter were calculated and plotted against the parameter for ease of comparison.

Fig. 2b. Levels of lipid peroxides (LPO) and 8-hydroxy-2-deoxyguanosine (8-OHdG) in type 2 DM and DF subjects in comparison with NC subjects. Values are statistically significant at *p<0.01. The type 2 DM and DF groups were compared with the non-diabetic NC group. The percentages of actual values of each parameter were calculated and plotted against the parameter for ease of comparison.
A slight increase in GPx activity was observed in the DM and DF groups (21.87% and 20.94% respectively) in contrast with a substantial decrease in SOD activity in the DF (4.09%) and especially in the DM group with a decrease of 14.82% (p<0.05) (Fig. 2c).

Discussion

During diabetes, persistent hyperglycaemia leads to an increased production of ROS through the glucose autoxidation, sorbitol pathway and non-enzymatic protein glycation. Oxidative stress arises when the production of ROS (which include both oxygen radicals such as superoxide (O•−), alkoxyl (RO), peroxy (ROO) and hydroxyl (HO) radicals, and non-radical derivatives of oxygen, namely hydrogen peroxide) exceeds the capacity of the available antioxidant defence system. The excess ROS tends to react with virtually all cell components, resulting in lipid membrane peroxidation, protein denaturation and DNA damage.2

In this study, a high level of HbA1c and FPG was found in both type 2 DM and DF groups, with the latter group having a greater increase as a consequence of poorly controlled glycaemia in these subjects. The correlation coefficient results obtained in this study revealed a strong association between these two parameters (FPG and HbA1c) and the level of oxidants and the activity of antioxidant enzymes in diabetic subjects with and without foot ulcers as well as non-diabetic subjects.

One of the characteristic features of chronic diabetes is lipid peroxidation resulting from excessive reactions of free radicals with polyunsaturated fatty acids (PUFAs) in cell membranes. This lipid peroxidation in turn leads to elevated production of free radicals.10 Lipid peroxide-mediated damage has been observed in both types of DM.

We have indeed confirmed in our study the findings of Santini et al.11 on the increase of plasma LPO levels in diabetic subjects, compared with those of control subjects. In addition to these observations, a substantially elevated level of this parameter was found in DF subjects, which may be due to their increased production of ROS.

As with other biomolecules, deoxyribonucleic acid (DNA) is also susceptible to damage induced by free radicals. An elevated level of 8-OHdG (a marker used for assessing the extent of DNA oxidative damage by free radicals) was indeed observed in type 2 DM and DF subjects (45.53% and 53.91% respectively), compared with the NC group. This finding is in agreement with the study by Dandona et al.,12 who reported greater oxidative damage to DNA with more increased generation of ROS in both type 1 and type 2 DM patients than normal controls.

Specific enzymatic antioxidant defence systems have evolved to deal with individual ROS. SOD scavenges superoxide radicals by converting them to hydrogen peroxide and molecular oxygen, while GPx converts hydrogen peroxide to oxygen and water.13

Controversial reports on changes in erythrocyte activity of SOD in both type 1 and type 2 diabetes have been published. In some, a decrease14 in the activity was observed, whereas in others an increase15 or no change16 was reported. In this study, however, a substantial decrease in SOD activity (14.82%; p<0.05) in DM and a slight reduction (4.09%; p>0.05) in DF groups were observed, compared with the NC group. The reduction in SOD activity observed in this study is compatible with the work of Bhatia et al.,4 who reported a significant reduction in SOD activity in DM subjects. The observed decrease in SOD activity could have resulted from glycation of the enzyme, which has been reported to occur in diabetes with poor glycaemic control.17

In addition, heterogeneous results of unchanged,14 decreased18 or elevated19 activity of erythrocyte GPx have been reported. In this study, a significantly elevated GPx activity was observed in DM (21.87%) and DF subjects (20.94%), compared with the NC subjects. These findings accord with the work of Gupta and Chari20 in both types of diabetes, where an increase was also reported. The increase in the activity of this alternative antioxidant enzyme may constitute a compensatory mechanism to prevent further tissue damage in diabetic subjects.

Conclusion

Findings in this study are compatible with the hypothesis that persistent hyperglycaemia leads to increased production of oxidants (LPO and 8-OHdG) in diabetic subjects. The increase is more pronounced in subjects with DF. Increased oxidation subsequent to diabetic conditions induces an over-expression of GPx activity, suggesting a compensatory mechanism by the body to prevent further tissue damage in such subjects.

We gratefully acknowledge the financial support of Third World Organization for Women in Sciences (TWOWS) and the technical assistance of the Chemical Pathology Department of the National Health Laboratory Services (NHLS) of South Africa.

References

Management of Type 2 Diabetes Mellitus, 2nd edition
A Practical Guide
By Steven Levene and Richard Donnelly
ISBN 0080449832 / 9780080449838 · Paperback · 312 Pages · 6 Illustrations
Butterworth Heinemann · Forthcoming Title (February 2008)
Price – R540.00
SAMA Member Price – R485.00

In the 21st Century, the management of type 2 diabetes has become even more important both in the primary health care setting and in the government’s health policy. With the publication of the National Service Framework and the allied National Clinical Guidelines, both patients and the government expect practices to deliver appropriate and effective care to a high standard. This book is aimed at all members of the primary health care team and concentrates on providing practical advice. Greater knowledge and skills will promote the delivery of high quality care to patients with type 2 diabetes in the community and, hopefully, reduce the impact of cardiovascular disease in this high risk group. This handbook addresses many concepts important in the day-to-day management of these patients. In addition to the discussion of specific medical management of type 2 diabetes (including the improvement of cardiovascular risk factors), the book explores the use of self-management techniques, the consultation process, and the use of psychological techniques to influence health-related behaviour. All aspects of the text are linked, when appropriate, to the GMS contract.

Features
- The authors include a full time GP delivering diabetic care and an eminent Consultant/academic at the leading edge of diabetes research - The text is completely up-to-date with numerous 2006 references, incorporating the latest guidance - The span of the text is comprehensive, including clinical, organisational and psycho-social topics of importance in delivering high-quality diabetes care - The text is cross-referenced to the relevant QOF indicators and NSF standards - This book also covers the relevant aspects of diabetes in Curriculum Statement 15.6 prepared by the Royal College of General Practitioners, which forms the basis of the new membership examination and the competencies expected of General Practitioners. - The management options include extensive balanced discussions about not just drugs, but also health education and appropriate referrals to specialists - The approach is neither didactic nor promotional, and aims to provide sufficient practical information to help clinicians make optimal decisions that take full account of the latest authoritative guidance, but which can be tailored rationally to the individual patient’s needs - Many of the concepts covered - including reduction of cardiovascular risk, health education, audit and lifestyle - are extremely relevant to non-diabetes care - The appendices include a detailed drug formulary and the relevant 2006-2008 QOF clinical indicators. Future trends and further reading are clearly set out, ensuring that the book will remain useful for the next few years.