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

Elizabeth Bosede Bolajoko, Kensese Sontin Mossanda, Francis Adeniyi, Olubayo Akinosun, Adesoji Fasanmade, Mpho Moropane

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 ulcer 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.

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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).

Department of Chemical Pathology, University of Limpopo, Medunsa Campus, GaRankuwa
Kensese Sontin Mossanda, PhD
Elizabeth Bosede Bolajoko, MSc

Department of Anatomical Pathology University of Limpopo, Medunsa Campus
Mpho Moropane, BTech

Department of Chemical Pathology, University of Ibadan, Ibadan, Nigeria
Francis Adeniyi, PhD
Olubayo Akinosun, MB BS

Department of Medicine, University of Ibadan
Adesoji Fasanmade, MB BS

Corresponding author: *K Mossanda (kmossan@ul.ac.za)*

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 xylenol orange (FOX VERSION II) assay according to the method of Nourooz-Zadeh *et al*.[5] This method is based on the principle of rapid peroxide-mediated oxidation of Fe_2^+ to Fe_3^+ under acidic conditions.

Plasma levels of 8-OHdG were measured at 450 nm on a microplate plate reader using a commercial kit from the Japan Institute for the Control of Aging (Fukuroi, Japan). 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.[6]

Erythrocyte SOD activity was determined by the method of Arthur and Boyne,[7] 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-nitrophenol)-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,[8] 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 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,

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

Correlation between test parameters		Pearson correlation coefficient ($p<0.05$)
NC FPG	NC LPO	0.919
NC FPG	NC 8-OHdG	0.977
NC FPG	NC SOD	–0.879
NC FPG	NC GPx	0.979
NC FPG	NC HbA1c	0.900
DM FPG	DM LPO	0.985
DM FPG	DM 8-OHdG	0.982
DM FPG	DM SOD	–0.915
DM FPG	DM GPx	0.926
DM FPG	DM HbA1c	0.982
DF FPG	DF LPO	0.842
DF FPG	DF 8-OHdG	0.928
DF FPG	DF SOD	–0.849
DF FPG	DF GPx	0.823
DF FPG	DF HbA1c	0.901
NC HbA1c	NC LPO	0.957
NC HbA1c	NC 8-OHdG	0.919
NC HbA1c	NC SOD	–0.710
NC HbA1c	NC GPx	0.908
DM HbA1c	DM LPO	0.965
DM HbA1c	DM 8-OHdG	0.975
DM HbA1c	DM SOD	–0.915
DM HbA1c	DM GPx	0.906
DF HbA1c	DF LPO	0.842
DF HbA1c	DF 8-OHdG	0.928
DF HbA1c	DF SOD	–0.849
DF HbA1c	DF GPx	0.823

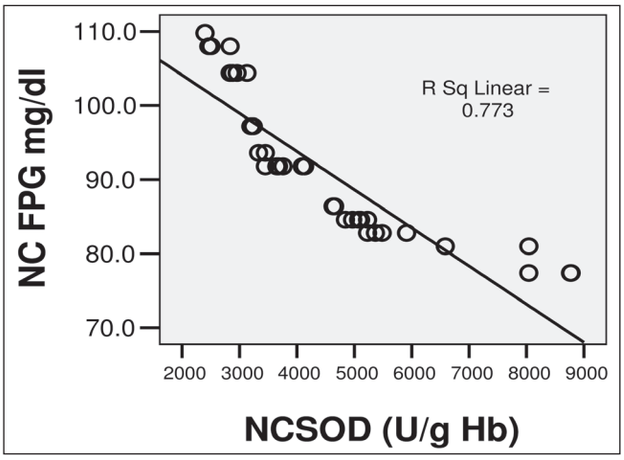

Fig. 1. Relationship between FPG and SOD in NC group. Coefficient of determination (R^2): –0.773; correlation coefficient (r): –0.879 (p<0.05).

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).

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,

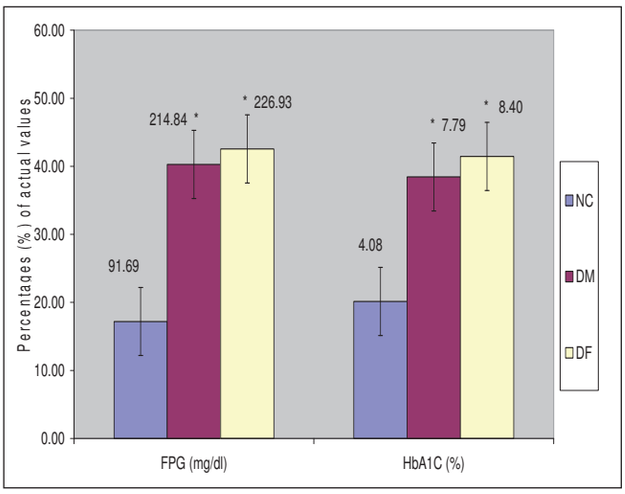

*Fig. 2a. Fasting plasma glucose (FPG) and glycated haemoglobin A1c (HbA1c) in type 2 DM and DF groups, compared with the NC group. Values are statistically significant at *p<0.01. The type 2 DM and DF groups were compared with the non-diabetic NC group. The percentages of actual values of each parameter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

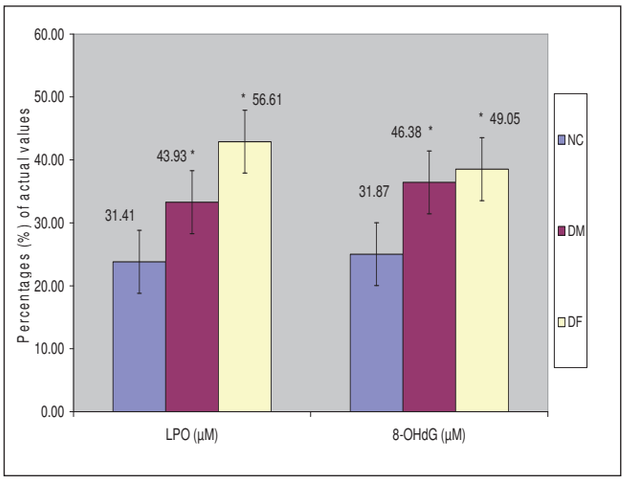

*Fig. 2b. Levels of lipid peroxides (LPO) and 8-hydroxy-2_-deoxyguanosine (8-OHdG) in type 2 DM and DF subjects in comparison with NC subjects. Values are statistically significant at *p<0.01. The type 2 DM and DF groups were compared with the non-diabetic NC group. The percentages of actual values of each parameter were calculated and plotted against the parameter for ease of comparison.*

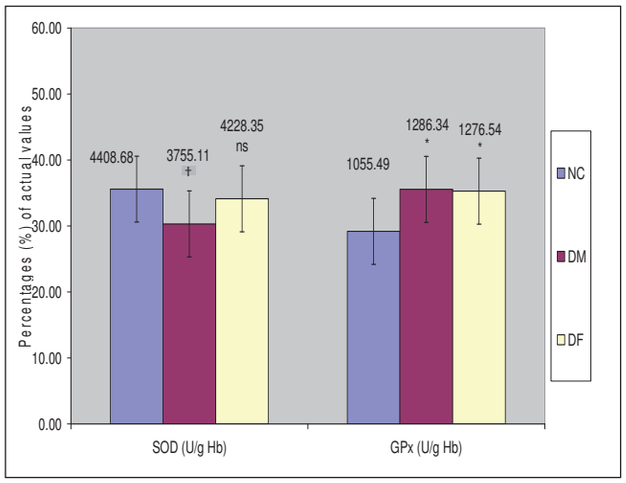

*Fig. 2c. The enzymatic activities of SOD and GPx in type 2 DM and DF subjects, compared with NC subjects. Values are statistically significant at *p<0.01 and †p<0.05, and not significant (ns) p>0.05. The type 2 DM and DF groups were compared with the non-diabetic NC group. Percentage of actual values of each parameter were calculated and plotted against the parameter for ease of comparison.*

sorbitol pathway and non-enzymatic protein glycation. Oxidative stress arises when the production of ROS (which include both oxygen radicals such as superoxide ($O_2\bullet-$), alkoxyl (RO), peroxyl (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.[9]

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[3]) 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 decrease[9] in the activity was observed, whereas in others an increase[14] or no change[15] 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 comparable to the work of Bhatia *et al*.,[9] 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.[13]

In addition, heterogeneous results of unchanged,[14] decreased[15] or elevated[16] 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 Chari[16] 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.

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