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8-hydroxy-2-deoxy-guanosine identifies oxidative DNA damage in a rural prediabetes cohort

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Running Title: Oxidative DNA damage in preclinical diabetes
Abstract

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Methods: Convenience sampling from people attending a diabetes screening clinic. Participants at the rural diabetes screening clinic had their medical history recorded as well as BMI, BGL, cholesterol, GSH, MDA, met-Hb and 8-OHdG measured. Statistical analysis was performed using ANOVA followed by Sheffe post hoc test for between group differences.

Results: 8-hydroxy-2-deoxy-guanosine (8-OHdG) level was significantly greater in the prediabetes (516.5 ± 260 pg/ml) compared to control group (177.8±91 pg/ml; p<0.01). The diabetes group (1926.9±1197 pg/ml) had the highest level of 8-OHdG, being approximately four times greater compared to the prediabetes group (p<0.001). No significant change in the cholesterol profile, MDA level indicative of lipid peroxidation and antioxidant activity as measured by erythrocyte reduced glutathione was observed in the prediabetes group compared to the control group (p>0.05).

Conclusion: 8-OHdG levels in both the prediabetes and diabetes group were increased from control values suggesting a role for 8-OHdG as an early disease marker that may be more sensitive compared to cholesterol, MDA and erythrocyte reduced glutathione levels, which were within normal limits. This is of clinical significance as 8-OHdG is a strong indicator of oxidative stress related DNA damage within blood vessel walls and other tissue that increases the risk of cardiovascular disease.

Keywords: 8-hydroxy-2-deoxy-guanosine, oxidative DNA damage, erythrocyte reduced glutathione, prediabetes, type 2 diabetes.
INTRODUCTION

Elevated blood glucose levels (BGL) lead to increases in free radicals via several biochemical pathways.\(^1\)\(^-\)\(^3\) An increase in oxidative stress and an impaired antioxidant capacity to retain normal physiological function play an important role in the pathogenesis of type 1 and type 2 diabetes mellitus and its complications such as atherosclerosis.\(^4\)\(^,\)\(^5\) Importantly, pathophysiological changes associated with diabetes complications such as diabetic retinopathy, and nephropathy may already be present at the preclinical stage when blood glucose levels are raised (5.5mmol/L < BGL < 7.0mmol/L) manifesting as changes in blood constituents associated with oxidative stress. These oxidative stress compounds include glutathione (GSH), malondialdehyde (MDA), methaemoglobin (metHb), interleukins, C-reactive protein (CRP), homocysteine and 8-hydroxy-2-deoxy-guanosine (8-OHdG).\(^6\)\(^-\)\(^9\) In diabetes disease progression, oxidative stress plays a key role in the development of insulin resistance and impaired insulin resistance.\(^10\) Early targeted treatment may reduce the likelihood of diabetes disease progression and increased occurrence of complications such as atherosclerosis.\(^11\)\(^,\)\(^12\)

Antioxidants and diabetes disease progression

Erythrocyte reduced glutathione (GSH) is a cellular antioxidant found in red blood cells.\(^13\) Patients with diabetes tend to have a smaller and more oxidized glutathione pool than control subjects of a similar age,\(^14\) which weakens the defence against oxidative stress.\(^15\) Hydrogen peroxide is a free radical that crosses into the nucleus and is a source of hydroxyl radicals, leading to oxidative damage of DNA. Hydrogen peroxide also crosses into the erythrocyte, where oxidative damage may occur. Hydrogen peroxide is primarily detoxified through glutathione activity located in plasma and within the erythrocyte.\(^16\) Erythrocytes of people with diabetes are also more susceptible to lipid peroxidation as measured by malondialdehyde in a TBARS reaction (MDA).\(^17\) The importance of MDA is that it reacts with proteins and phospholipids, especially with collagen within the cardiovascular system, which in diabetes
has already undergone modification due to increases in glucose and glycation products. The stiffened collagen becomes increasingly more resistant to remodelling and is a contributor to atherosclerosis and heart attack.\textsuperscript{18} Methaemoglobin (met-Hb), where the iron in haemoglobin is oxidized to the ferric form, is unable to transport oxygen to tissues causing hypoxia.\textsuperscript{19} Both oxidative stress induction and stress adaptation are significantly reduced if haem release from methaemoglobin is inhibited.\textsuperscript{20}

\textbf{8-OHdG and diabetes disease progression}

Oxidative damage through free radicals formed through oxidation of hydrogen peroxide in the nucleus can lead to base modification, sugar damage, strand break, and DNA-protein cross-links.\textsuperscript{21} 8-hydroxy-2-deoxy-guanosine (8-OHdG), a product of DNA base modification at the C-8 site produced by the oxidation of deoxyguanosine, is considered as the most sensitive and useful biomarker of oxidative DNA damage.\textsuperscript{22, 23}

This research compared the extent of oxidative damage present in control, prediabetes and diabetes stage by measuring 8-OHdG levels, cholesterol levels, erythrocyte reduced glutathione, erythrocyte malondialdehyde and methaemoglobin.

\textbf{MATERIALS AND METHODS}

\textit{Protocol}: The study protocol was reviewed and approved by the Ethics in Human Research Committee of Charles Sturt University. Informed consent was obtained from each subject. Participants to the Diabetes Screening Clinic were drawn from the community through announcements in the local newspaper, radio and television. During the period of the study between February 2006 and June 2008, 753 people attended the clinic. Only participants where complete data was available as required for this study were included in the analysis. Participants were divided into three groups: a) Control group with normal blood glucose
levels. b) Prediabetes group with impaired fasting blood glucose (BGL > 5.5 mmol/L but < 7 mmol/L) according to the recommendations of the American Diabetic Association;\textsuperscript{24} and c) Diabetes group, selected on the basis of having been diagnosed previously using an oral glucose tolerance test and/or being on antihyperglycaemic medication. No exclusions were applied for the prediabetes and diabetes group as cardiovascular and/or renal disease are a part of the disease progression. Medication that has antioxidant properties is expected to reduce the level of antioxidants measured and therefore would reduce the likelihood of seeing a difference between groups. Participants were comparable for age, gender, smoking habit, diet, and physical activity. People with type 2 diabetes were requested not to take any diabetic medication on the day of the test.

*Measurements of oxidative stress.* Erythrocyte malondialdehyde (MDA) was measured using the thiobarbituric acid reacting substance.\textsuperscript{25} Levels of methaemoglobin (MetHb) were assessed using spectrophotometry of haemoglobin absorption before and after cyanide addition,\textsuperscript{26} and oxidative DNA damage was measured using the serum 8-Hydroxy 2'-deoxy Guanosine ELISA Kit.\textsuperscript{27} The level of erythrocyte reduced glutathione (GSH) was determined using the 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) reaction.\textsuperscript{28,29}

*Statistical analysis.* The data was analysed using SPSS (Version 14) and Microsoft Excel (Office2007, Microsoft). All values were expressed as mean ± standard deviation (M±SD). Statistical analysis was performed using a one-way ANOVA followed by Scheffe post-hoc test for between group comparisons. For categorical variables a chi-square test was used.
RESULTS

Table 1 illustrates the baseline values of 166 participants. Disease status, fasting blood glucose level, age, sex, HbA1c, blood pressure, statin therapy and BMI are shown. Presence of microvascular and macrovascular disease is also indicated.

PUT TABLE 1 HERE

Thirty-six of the control group (36.7%) had medically treated hypertension and 18 (18.4%) were receiving statin. In the prediabetes group twelve (36.4%) had hypertension and receiving medication and eleven (33.3%) were receiving statins. While 27 (77.1%) of the diabetes subjects were receiving medication for hypertension and 20 (57.1%) were on statin therapy. No significant difference between the three groups was found for use of statins ($\chi^2_{166,2} = 2.73; p=0.25$). There was a significant difference for antihypertensive use ($\chi^2_{166,2} = 2.73; p<0.0001$) One control subject reported glomerular nephritis, Nineteen (8 control, 5 pre-DM and 6 DM) had other kidney complaints such as kidney stones and cysts. Participants were comparable for age, gender, smoking habit, diet, and physical activity. Age, BMI, blood pressure, BGL and HbA1c increased from the control to the prediabetes and diabetes groups. Using post hoc analysis, BMI was significantly different between control and diabetes group (Table 1). BGL and HbA1c showed significant changes between control and diabetes and BGL between prediabetes and control as well.

For the biomarkers a statistically significant result for total cholesterol (TC), high and low density lipoprotein cholesterol (LDL-C; HDL-C) as well as triglycerides between the three groups was noted using the one way ANOVA ($p < 0.001$). Similarly reduced GSH and 8-OHdG were significantly different ($p < 0.001$).
There was a significant elevation of 8-OHdG in the prediabetes (516.5 ± 260 pg/ml) and diabetes (1926.9 ± 1197 pg/ml) groups (p<0.01 and p<0.001 respectively) compared to the control group (177.8 ± 91.1 pg/ml) (Table 2) using the Sheffe post hoc test. In addition, there was also a significant higher 8-OHdG level for the diabetes compared to the prediabetes group (p<0.001).

A reduction in reduced GSH levels occurred with disease progression from control to prediabetes and diabetes. However the Scheffe post hoc test only showed a statistically significant difference between GSH level in the control (71.9 ± 10.4 mg/100ml) and the diabetes group (61.5 ± 14 mg/100ml) (p < 0.001). The reduction noted for reduced GSH level for the prediabetes group (66.9 ± 20.4 mg/100ml) compared to the control group was not statistically significant (Table 2).

Post hoc Sheffe analysis for between group differences indicated a statistically significant difference between the control and diabetes group for all cholesterol parameters (p<0.001). The level of total cholesterol was found to be lower in the type 2 diabetes group (4.4 ±1.2 mmol/L) compared to the prediabetes and control group (5.1±1.2 mmol/L and 5.16±0mmol/L respectively). There was also a significant reduction in the level of LDL-C in the type 2 diabetes group (2.4±1 mmol/L) compared to the prediabetes group (3.19±0.1 mmol/L) and control group (3.26±0.8 mmol/L); p values were 0.001 & 0.01 respectively (Table 3).
DISCUSSION

Reactive oxygen species (ROS), which arise as a result of hyperglycaemia, can damage nucleic acids, lipids, and proteins with parallel changes in the biochemical makeup of blood constituents. Increased levels of biomarkers or risk factors associated with oxidative damage to lipids, proteins and DNA have been detected in serum of diabetic patients and their presence is correlated with the development of diabetes complications including atherosclerosis.\textsuperscript{30,31} The extent of damage is related to the duration and level of hyperglycaemia, where even small elevations of blood glucose, as is the case for the prediabetes group (BGL > 5.5 mmol/L) may lead to tissue damage. Thus long-term elevated blood glucose levels are in part responsible for progression of diabetes complications including micro and macrovascular disease.\textsuperscript{32} It is well known that complications of diabetes occur often prior to diagnosis of diabetes such as diabetic retinopathy or nephropathy with early intervention being of clear benefit.\textsuperscript{33}

Elevated blood glucose levels are clearly responsible for microvascular complications of diabetes and the pathogenesis of atherosclerotic macrovascular disease.\textsuperscript{34} Hyperglycaemia leads to the formation of reactive oxygen species. These superoxide anions cause DNA strand breakage with an increase in 8-OHdG and destruction of endothelial function resulting in atherosclerosis.\textsuperscript{1} Erythrocyte glutathione or reduced glutathione is a marker of oxidative stress, where free radicals entering the red blood cell are scavenged by glutathione, which is reduced. The red blood cell is thus protected and haemoglobin can continue to function at optimal capacity.\textsuperscript{9}

Setting the blood glucose level at 5.5mmol/L for identification of prediabetes has increased the sensitivity of detecting people at risk of diabetes and cardiovascular disease but also has
increased the number that need to be reviewed.\textsuperscript{35} Thus it becomes important to easily identify those subjects who would benefit most from intervention.

Our study showed a statistically significant increase in the level of 8-OHdG for the prediabetes group, which was even greater in the diabetes group. Since diverse pathologies affect 8-OHdG level especially nephropathy we also examined the level of kidney disease in our cohort. Only one person in the control group had glomerular nephritis but with 8-OHdG levels within the range of the control group. Therefore the level of 8-OHdG in the first instance indicates oxidative stress and DNA damage due to the glycaemic state of the participants. As the prediabetes group has higher glucose levels compared to the control group, the resulting ROS formed will lead to more oxidative DNA damage.\textsuperscript{1} The rise in 8-OHdG in the prediabetes group is a strong indicator that not only oxidative damage is occurring but that possible microvascular (retinopathy or nephropathy) and/or macrovascular (cardiovascular disease or peripheral vascular disease) disease is already present subclinically.\textsuperscript{5,30} 8-OHdG may therefore be a useful indicator of oxidative stress and associated tissue/organ damage much earlier in disease progression. That is, our prediabetes cohort, although asymptomatic for micro and macrovascular disease, with BGL and HbA1c level in the normal range, had a statistically significant increase in 8-OHdG compared to the control group. Hinoko et al. reported an increase in 8-OHdG associated with hyperglycaemia in people with diabetes and our study now extends this to the prediabetes stage and confirms the results of Kopprasch et al. and Deedwania & Fonseca,\textsuperscript{11,33} who suggested that impaired glucose tolerance and/or impaired fasting glucose contribute to the risk of future cardiovascular disease. Increased oxidative stress as shown by the increase in 8-OHdG and decreased GSH may be related to the pathogenesis of diabetes complications.\textsuperscript{36} It is of interest that the HbA1c levels between the control and prediabetes group were not significantly different and in fact within normal limits. HbA1c values for the diabetes group
were elevated but still indicating reasonable control of diabetes. Therefore relying solely on glycated haemoglobin as an indicator of risk for progression of diabetes complications may not be warranted.

Clinical relevance of elevated 8-OHdG levels are supported by previous studies with increases in 8-OHdG noted in urine and muscle in type 2 diabetes in accordance with disease progression and DNA damage in leukocytes of type 2 diabetic patients compared to control subjects.\textsuperscript{30,37,38} From this point of view, it is important to evaluate the severity of oxidative stress associated with the loss of normal glycaemic control using 8-OHdG as similar changes were not seen for erythrocyte malondialdehyde and methaemoglobin. The minimum 8-OHdG levels recorded remained similar for all three groups. This is expected as some people with prediabetes or diabetes will not have pathophysiological changes associated with diabetes at the time of the study. Importantly the sensitivity of 8-OHdG is such that we observed a significant elevation in the prediabetes group compared to control group despite good HbA1c readings in all groups. An elevation of 8-OHdG indicates an increase in the degree of oxidative stress affecting tissue function and integrity, and therefore provides useful information.\textsuperscript{39} Initial increases in 8-OHdG may be associated with the guanine base of DNA being more susceptible to oxidative damage due to its inherent chemical structure.\textsuperscript{40} GSH is not increased significantly in the prediabetes group due to a slower response dependent on more factors such as amount of GSH stored in erythrocyte balanced by de novo synthesis, which is balanced during the prediabetes stage. DNA damage in the erythrocyte is independent to vessel pathology associated with increases in LDL-cholesterol due to the free radical initiated pathophysiology. Increased 8-OHdG may also be seen earlier as a protective mechanism by DNA to increases in free radicals as part of the rapid oxidation of the guanosine nucleotide and cellular anti-inflammatory response occur prior to changes in GSH and cholesterol profile being observed.\textsuperscript{41}
Our study indicates a rise in 8-OHdG level from control to prediabetes and diabetes stage. 8-OHdG is an expression of DNA damage in endothelial tissue and oxidative stress. The erythrocyte GSH levels are lower in the prediabetes group and lowest in the diabetes group. This latter finding confirms oxidative stress and free radical activity being increased with diabetes disease progression. GSH is used up in the erythrocyte to bind with the free radicals and therefore a decrease is observed. As free radical activity increases GSH levels in the erythrocyte tend to increase as there is de novo synthesis occurring until free radical damage such as erythrocyte membrane transporter for cysteine and a lack of cysteine due to increases in homocysteine lead to a reduction in GSH.8, 9

There was no significant elevation in erythrocyte malondialdehyde and methaemoglobin in the prediabetes and type 2 diabetes groups compared to the control group. These results are not what we expected especially for MDA, which is an early marker for atherosclerosis in association with low density lipoprotein. However, cholesterol levels may have been affected due to the statin medication taken by the majority of the diabetes group, which was not stopped for this research and therefore the LDL-C values decreased with disease progression (Table 3). In addition the concentration of MDA by the thiobarbituric acid assay in untreated serum may not reflect free radical damage to lipoproteins.42 MetHb is a late marker for oxidative stress and is formed once other antioxidants such as GSH are non-functional in advanced diabetes and therefore no change may have occurred at this stage of disease progression in our cohort.

Dancer et al.43 reported a negative correlation between fasting blood glucose and reduced glutathione levels in patients with type 2 diabetes. Similarly our results indicate that there was a significant reduction in GSH in the type 2 diabetes group compared to the control subjects with a lower level in the prediabetes group compared to control, (Table 2). This reduction in erythrocyte reduced glutathione in type 2 diabetic patients is due to its action as
an antioxidant against ROS generated during oxidative stress.\textsuperscript{4,9,31} At the diabetes stage the further decrease in GSH may now be associated with reduction of existing GSH reserves in the erythrocyte without replenishment or increased production. Normal erythrocyte function replenishes GSH by chemical reactions using cysteine transported across the membrane from the plasma.\textsuperscript{44} However in diabetes progression free radical damage to the endothelium leads to an increase in homocysteine, which decreases available cysteine to be transported into the erythrocyte for GSH production and the erythrocyte membrane cysteine transport is also impaired.

**CONCLUSION**

Our results indicate that 8-OHdG is increased at the prediabetes stage suggesting that free radical damage associated with minor increases in blood glucose level has occurred. Reduced erythrocyte GSH is an antioxidant marker, which is slightly decreased in the prediabetes and further reduced in the diabetes group. This latter finding suggests the role of the erythrocyte in combating oxidative stress is already compromised at the prediabetes stage. 8-OHdG could be used as a biomarker for oxidative stress related DNA damage that is associated with an increased risk of cardiovascular disease when blood glucose levels are raised but below the diabetes cut-off.

**ACKNOWLEDGEMENTS**

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REFERENCES:


### TABLE 1

**Characteristics of the subjects included in this study**

<table>
<thead>
<tr>
<th></th>
<th>Control subjects</th>
<th>Prediabetic Subjects</th>
<th>Type 2 Diabetic Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>98</td>
<td>33</td>
<td>35</td>
</tr>
<tr>
<td>Age (years)</td>
<td>66.2 ± 11.2</td>
<td>64.7 ±10.4</td>
<td>70 ± 8.4</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>40/58</td>
<td>16/17</td>
<td>20/15</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>27.2 ± 4.6*</td>
<td>29.1 ± 4.4</td>
<td>31.1 ± 4.9</td>
</tr>
<tr>
<td>Fasting Plasma Glucose (mmol/L)</td>
<td>4.8 ± 0.5*</td>
<td>6.1 ± 0.9**</td>
<td>7.2 ± 3.2</td>
</tr>
<tr>
<td>HbA1c %</td>
<td>5.6 ± 0.26*</td>
<td>5.7 ± 0.27**</td>
<td>6.7 ± 1.1</td>
</tr>
<tr>
<td>Systolic Blood Pressure (mmHg)</td>
<td>130.1 ± 19.7</td>
<td>129.9 ± 15.3</td>
<td>135.3 ± 15</td>
</tr>
<tr>
<td>Diastolic Blood Pressure (mmHg)</td>
<td>76.6 ± 8.6</td>
<td>77.1 ± 8.6</td>
<td>74.6 ± 10.1</td>
</tr>
<tr>
<td>Nephropathy</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Statin</td>
<td>18 (18.4%)</td>
<td>11 (33.3%)</td>
<td>20 (57.1%)</td>
</tr>
<tr>
<td>Antihypertensive Medication</td>
<td>36 (36.7%)</td>
<td>12 (36.4%)</td>
<td>27 (77.1%)</td>
</tr>
<tr>
<td>Single medication</td>
<td>29</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Two medications</td>
<td>6</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>Three medications</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>More than three medications</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

*significant difference between control and type 2 diabetes (p<0.001)

#significant difference between control and prediabetes (p<0.001)

**significant difference between prediabetes and type 2 diabetes (p<0.05)
**TABLE 2**

**Biomarkers of Oxidative Stress (Mean ± SD) in Control, Prediabetic and Diabetic groups**

<table>
<thead>
<tr>
<th>Oxidative Stress Biomarkers</th>
<th>Control Group</th>
<th>Prediabetes Group</th>
<th>Type 2 Diabetes Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH (mg/100ml)</td>
<td>71.9 ± 10.4*</td>
<td>66.9 ± 20.4</td>
<td>61.5 ± 14</td>
</tr>
<tr>
<td>8-OHdG (pg/ml)</td>
<td>177.8 ± 91.1*,#</td>
<td>516.5 ± 260**</td>
<td>1926.9 ± 1197</td>
</tr>
</tbody>
</table>

*significant difference between control and type 2 diabetes (p<0.001)

#significant difference between control and prediabetes (p<0.01)

**significant difference between prediabetes and type 2 diabetes (p<0.001)

GSH (erythrocyte reduced glutathione); 8-OHdG (8-OH-2-deoxy-Guanosine)
TABLE 3
Serum Lipid Profile (Mean ± SD) in Control, Prediabetic and Diabetic groups (mmol/L)

<table>
<thead>
<tr>
<th>Serum Lipid Profile</th>
<th>Control Group</th>
<th>Prediabetes Group</th>
<th>Type 2 Diabetes Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol</td>
<td>5.16 ± 1*</td>
<td>5.1 ± 1.2</td>
<td>4.4 ± 1.2</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.2 ± 0.6*</td>
<td>1.5 ± 0.9</td>
<td>1.9 ± 1.02</td>
</tr>
<tr>
<td>HDL-C</td>
<td>1.35 ± 0.3*</td>
<td>1.31 ± 0.3</td>
<td>1.12 ± 0.1</td>
</tr>
<tr>
<td>LDL-C</td>
<td>3.26 ± 0.8*</td>
<td>3.19 ± 0.1**</td>
<td>2.4 ± 1</td>
</tr>
</tbody>
</table>

HDL-C high density lipoprotein cholesterol; LDL-C low density lipoprotein cholesterol.

*significant difference between control and type 2 diabetes group (p<0.01)

**significant difference between prediabetes and type 2 diabetes group (p<0.001)