Oxidative stress, antioxidant status and DNA damage in patients with impaired glucose regulation and newly diagnosed Type 2 diabetes

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ABSTRACT

Previous studies have postulated the association between oxidative stress and Type 2 diabetes. Considering the long pre-diabetic period with IGR (impaired glucose tolerance) and its high risk of developing diabetes, to test this hypothesis, we have investigated oxidative stress pathways and DNA damage in patients with IGR and newly diagnosed Type 2 diabetes. The study population consisted of 92 subjects with NGT (normal glucose tolerance), 78 patients with IGR and 113 patients with newly diagnosed diabetes. Plasma MDA (malondialdehyde) and TAC (total antioxidative capacity) status, erythrocyte GSH content and SOD (superoxide dismutase) activity were determined. A comet assay was employed to evaluate DNA damage. Compared with subjects with NGT, patients with IGR had reduced erythrocyte SOD activity. Patients with diabetes had a higher plasma MDA concentration, but a lower plasma TAC level and erythrocyte SOD activity, than the NGT group. Correlation analysis revealed a strong positive association between IR (insulin resistance) and MDA concentration, but negative correlations with TAC status and SOD activity. With respect to β-cell function, a positive association with TAC status and an inverse correlation with GSH respectively, were observed. The comet assay revealed slight DNA damage in patients with IGR, which was increased in patients with diabetes. Significant correlations were observed between DNA damage and hyperglycaemia, IR and β-cell dysfunction. In conclusion, the results of the present study suggest that hyperglycaemia in an IGR state caused the predominance of oxidative stress over antioxidative defence systems, leading to oxidative DNA damage, which possibly contributed to pancreatic β-cell dysfunction, IR and more pronounced hyperglycaemia. This vicious circle finally induced the deterioration to diabetes.

Key words: antioxidant, comet assay, DNA damage, glucose tolerance, impaired glucose regulation, oxidative stress, Type 2 diabetes.

Abbreviations: BMI, body mass index; BP, blood pressure; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); FPG, fasting plasma glucose; FPI, fasting plasma insulin; HbA1c, glycated haemoglobin; HOMA, homeostasis model assessment; HOMA-β-cell, HOMA of β-cell function; IFG, impaired fasting glucose; IGR, impaired glucose regulation; IGT, impaired glucose tolerance; IR, insulin resistance; LMA, low-melting-point agarose; MDA, malondialdehyde; m-units, milliunits; NGT, normal glucose tolerance; NMA, normal-melting-point agarose; OGTT, oral glucose tolerance test; 8-OHdG, 8-hydroxy-2-deoxyguanosine; OTM, olive tail moment; PBMC, peripheral blood mononuclear cell; SOD, superoxide dismutase; TAC, total antioxidative capacity.

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INTRODUCTION

Type 2 diabetes is a well-known endocrine and metabolic disorder which has reached epidemic proportions worldwide and represents a serious public health concern. It is estimated that it will affect approx. 366 million people by 2030 [1], and another sobering fact is that this epidemic increases at a striking rate in children and adolescents [2], with the biggest increases in developing countries, such as India and China [3]. Currently in China, Type 2 diabetes affects up to 20.8 million people, and this figure is expected to approach 42.3 million by 2030 [1]. Considering its devastating effects on mortality and morbidity, various and combined drug therapies and lifestyle interventions have been applied to prevent or delay the progression of diabetes; however, this epidemic will probably continue to grow over the next 20–30 years, suggesting an urgent need to explore new therapeutic strategies.

Type 2 diabetes is characterized by defective insulin secretion in pancreatic β-cells in response to glucose and by deficiencies in insulin action on its target tissues. Indeed, the relative importance of β-cell dysfunction has been reported in clinical studies [4–6]. In a state of IR (insulin resistance), normal pancreatic β-cells can compensate for insulin insensitivity by up-regulating insulin secretion; however, insufficient secretion by β-cells can induce the onset of abnormalities in glucose metabolism, i.e. IGR (impaired glucose regulation) [5]. Once hyperglycaemia becomes evident, the function of pancreatic β-cells deteriorates progressively due to ‘glucose toxicity’, which leads to severe impairment of glucose-stimulated insulin secretion, apparent degranulation of β-cells and decreased β-cell number, resulting in the aggravation of IR. This vicious circle finally results in the clinical manifestation of diabetes [7,8].

Obviously, the natural progression of diabetes occurs after a prolonged pre-diabetic period, during which IGR forms an important intermediate and reversible stage [9]. This abnormal metabolic state between NGT (normal glucose tolerance) and diabetes consists of two distinct disorders: IFG (impaired fasting glucose) and IGT (impaired glucose tolerance). Compared with subjects with NGT, patients with IFG or IGT, unless treated, have a considerably higher risk of developing diabetes and cardiovascular disease [9–11] and, thus, can be used as a significant target group for the primary prevention of Type 2 diabetes. However, the question as to whether the onset of Type 2 diabetes in patients with IGR can be prevented or delayed has been poorly addressed over the last few years.

Although the underlying mechanisms of the pathogenesis of Type 2 diabetes still remain to be determined, oxidative stress has been shown to be responsible, at least in part, for the progression of Type 2 diabetes [12] and is supported by increased oxidative damage to lipids and DNA and impaired antioxidative defence systems in these patients [13], as well as the effectiveness of some antioxidants used as adjuvants in the treatment of Type 2 diabetes [14,15]. However, it still remains to be elucidated whether oxidative stress acts as a prime cause, just emerges as a concomitant event or is a consequence of the onset of diabetes. Furthermore, most research into Type 2 diabetes currently focuses on patients in a hospital setting, and results from these studies possibly do not reflect the exact metabolic changes and oxidative stress state in the progression of Type 2 diabetes, due to the latent interference from improved lifestyles and drug therapy guided by hospital staff. These findings have stimulated us to search for objective biomarkers of oxidative stress in patients with different states of glucose metabolism, including IFG, IGT and newly diagnosed Type 2 diabetes, and to explore the inter-relationships between oxidative stress status, IR and pancreatic β-cell secretory function in an attempt to investigate the role of oxidative stress in the aetiology of Type 2 diabetes, which may have important implications for the prevention of Type 2 diabetes in pre-diabetic patients.

MATERIALS AND METHODS

Subjects

We studied 78 patients with IGR and 113 patients with newly diagnosed Type 2 diabetes, according to the well-established diagnostic criteria as recommended by the World Health Organization incorporating both fasting glucose and a 2-h OGTT (oral glucose tolerance test; 75 g of glucose) [i.e. Type 2 diabetes, FPG (fasting plasma glucose) \(\geq 7.0 \text{ mmol/l} \) and/or 2-h post-glucose load \(\geq 11.1 \text{ mmol/l} \); IGT, FPG (if measured) < 7.0 mmol/l and 2-h post-glucose load \(\geq 7.8 \text{ mmol/l} \) and < 11.1 mmol/l; and IFG, FPG \(\geq 6.1 \text{ mmol/l} \) and < 7.0 mmol/l and 2-h post-glucose load (if measured) < 7.8 mmol/l] [5]. The subjects were recruited consecutively from those attending the outpatient clinics at the Department of Endocrinology, Tongji Medical College Hospital, Wuhan, People’s Republic of China. The inclusion criteria of patients with IGR and newly diagnosed diabetes were: age \(\geq 30\) years, BMI (body mass index) < 40 kg/m², no history of a diagnosis of diabetes and no history of receiving pharmacological treatment for hyperlipidaemia and hypertension. Patients with clinically significant neurological, endocrinological or other systemic diseases, as well as acute illness and chronic inflammatory or infective diseases, were excluded from the study. An age- and sex-matched NGT group consisting of 92 unrelated subjects was recruited from an unselected population undergoing routine health check-ups at Tongji Medical College Hospital, Wuhan, People’s Republic of China. The enrolment criteria for subjects with NGT were: no family history of diabetes in their first-degree relatives,
FPG concentration < 6.1 mmol/l, a 2-h OGTT plasma glucose concentration < 7.8 mmol/l, HbA1c (glycated haemoglobin) < 5.6 %, no past history of IGR or diabetes, and the absence of any systemic disease or infections in the previous months.

All of the subjects enrolled in the present study were of Chinese ethnicity. The Ethics Committee of Tongji Medical College approved the study protocol, and written informed consent to participate in the study was obtained from all the individuals.

A standard questionnaire was designed to collect information about age, sex, history of disease (diabetes, hyperlipidaemia and hypertension), family history of diabetes, diet habits, tobacco consumption and physical activity. All of the study subjects underwent a complete physical examination in the morning after an overnight fast. Anthropometric parameters, including weight and height as well as BP (blood pressure), were measured, and BMI was calculated [weight (kg)/square of height (m²)].

**Methods**

Venous blood samples from all the participants were drawn after an overnight fast from an antecubital vein into heparinized tubes. Plasma was separated and retained for analysis of biochemical parameters [FPG, FPI (fasting plasma insulin), HbA1c, total cholesterol and triacylglycerol (triglyceride)] and for estimation of MDA (malondialdehyde) and TAC (total antioxidative capacity). Subsequently, PBMCs (peripheral blood mononuclear cells) were isolated by Ficoll–Hypaque density gradient centrifugation, according to the manufacturer’s protocol (TBD). Briefly, the remaining blood was returned to its original volume with PBS, mixed and then gently added to tubes containing Lymphocyte Separation Medium (TBD), followed by centrifugation at 720 g for 20 min at room temperature (25 °C). The PBMC-containing band was aspirated, washed with PBS and analysed microscopically. The cell number was counted and adjusted to 1 × 10⁶/ml for the comet assay. The residual erythrocyte pellets were collected, washed with 0.9 % NaCl and lysed in an appropriate volume of double-deionized water for the determination of haemoglobin and HbA1c, GSH concentration and SOD activity. Erythrocyte lysates were stored at −20 °C until analysed, whereas the comet assay was performed immediately after PBMCs were separated.

All of the reagents used were of analytical grade. GSH, DTNB [5,5’-dithiobis-(2-nitrobenzoic acid)], thiorbarbituric acid, 1,1,3,3-tetraethoxypropane, xanthine, xanthine oxidase, hydroxylamine, sulfanilic acid, naphthyl ethylenediamine and TPTZ (2,4,6-tripyridyl-s-triazine) were from Sigma–Aldrich. NMA (normal-melting-point agarose) and LMA (low-melting-point agarose) were obtained from Biowest and Gibco respectively. Tris base, DMSO, sodium sarcosinate, EDTA, Trixon X-100 and ethidium bromide were from Amresco.

**Baseline biochemical analysis**

The cyanomethemoglobin method was employed to estimate the haemoglobin concentration using Drabkin’s reagent, and HbA1c was determined by HPLC using a Daini Glycosylated Hemoglobin Analyser (Bio-Rad Laboratories) and was expressed as a percentage of glycosylated haemoglobin. FPG, total cholesterol and triacylglycerol were analysed by colorimetric enzymatic methods using commercial kits (Zhongsheng Beikong Bio-Technology and Science). The FPI concentration was estimated in duplicate using an electrochemiluminescence immunoassay kit (Roche Diagnostics) and was performed in an automated Elecsys 2010 immunoassay analyser (Roche), using a double-antibody system with 0.05 % cross-reactivity between insulin and its precursor (pro-insulin).

The IR index was evaluated by the HOMA (homoeostasis model assessment)-IR score using the formula: FPI [m-units (milliunits)/l] × FPG (mmol/l)/22.5, and has excellent correlation with the euglycaemic hyperinsulinemic clamp [16,17]. HOMA-β-cell (HOMA of β-cell function) was calculated as 20 × FPI (m-units/l)/[FPG (mmol/l) − 3.5][16].

**Analysis of lipid peroxidation and antioxidant status**

**Determination of lipid peroxidation**

MDA was measured as an index of lipid peroxidation using the method described by Beuege and Aust [18]. Briefly, plasma samples (0.3 ml) were mixed with a trichloroacetic acid/thiobarbituric acid/HCl reaction solution (5:2:1, by vol.), heated for 60 min in a boiling water bath, cooled with tap water and centrifuged. The absorbance of the supernatant was recorded spectrometrically at 532 nm with 1,1,3,3-tetraethoxypropane as a standard (results are expressed as nmol MDA formed/ml of plasma).

**Estimation of erythrocyte GSH content**

Erythrocyte GSH content was estimated using the method described by Beutler et al. [19]. Protein in erythrocyte lysates was precipitated with metaphosphoric acid and removed after centrifugation at 3000 g for 15 min. Subsequently, 0.3 mol/l Na₂H₂PO₄ and 1 mmol/l DTNB solution were added and the absorbance was read at 412 nm. A series of GSH standards were also run to give a standard curve for the determination of GSH in the samples (results are expressed as mg/g of haemoglobin).

**Erythrocyte SOD activity**

The activity of SOD in erythrocyte lysates was based on its ability to inhibit the oxidation of hydroxylamine, as described previously [20], using a xanthine/xanthine oxidase system.
oxidase system to generate superoxide radicals, which oxidize hydroxylamine to nitrite. The latter reacts with sulfanilic acid and naphthyl ethylenediamine to form a red chromophore. A chloroform/ethanol [1:1 v/v] extract of erythrocyte haemolysates was prepared and the supernatant containing this enzyme was used for the enzyme assay. One unit of SOD was defined as the amount of SOD causing 50% inhibition of hydroxylamine oxidation/ml of reaction solution (results are expressed as units/mg of haemoglobin).

Assay of plasma TAC
TAC in the plasma was measured on the basis of the ability of antioxidants in the samples to reduce Fe\(^{3+}\)-TPTZ to Fe\(^{2+}\)-TPTZ, a stable blue product proportional to the TAC, which was measured at 593 nm [21]. One unit of TAC is represented by an increase in the absorbance of the reaction mixture of 0.01 per ml of plasma per min (results are expressed as units/ml of plasma).

Comet assay
In samples from a subset of subjects from the study population (27 subjects with NGT, 16 patients with IGR and 34 patients with diabetes), DNA damage in PBMCs was assessed using the comet assay method, a well-validated technique developed for measuring DNA-strand breaks in individual cells, as described by Singh et al. [22] and Tice et al. [23].

Slide preparation
Frosted microscopic slides were pre-coated with 0.5% NMA dissolved in PBS as the first layer, covered immediately with a large coverslip and kept at room temperature until the agarose had solidified. After removal of the coverslip, a mixture of PBMC suspension and 0.5% LMA was rapidly added on top of the first agarose layer, and was maintained on a flat tray at 4°C for solidification. Finally, a third layer of 0.5% LMA was added. The slides were immersed in pre-cooled high-salt lysing buffer [2.5 mol/l NaCl, 100 mmol/l EDTA, 10 mmol/l Tris base and 1% sodium sarcosinate (pH 10) containing freshly added 1% Triton X-100 and 10% (v/v) DMSO] at 4°C for 1 h to remove the cell membrane and soluble contents of the cytoplasm and nucleoplasm, except nuclear material.

Electrophoresis, neutralizing and staining
After lysis, the slides were placed in an electrophoresis tank filled with freshly prepared electrophoresis buffer (300 mmol/l NaOH and 1 mmol/l EDTA) for 45 min to allow DNA supercoils to unwind from sites of strand breakage before electrophoresis. Electrophoresis was then conducted on ice with an electric field of 25 V and 300 mA for 20 min. All of the above steps were carried out in a dark room to prevent the interference of additional DNA damage. Subsequently, the slides were gently removed from the electrophoresis tank, washed with the neutralizing buffer [0.4 mol/l Tris/HCl (pH 7.5)], stained with ethidium bromide and examined within 24 h.

Microscopic examination and analysis
The slides were examined at ×200 and ×400 magnifications using a fluorescence microscope (Olympus) equipped with an excitation filter of 549 nm and barrier filter of 590 nm, and attached to a video camera (Olympus). Two parallel tests on the same cell suspension were performed on each slide, and images of 100 cells selected randomly were captured and analysed using comet assay image analysis software (Casp 1.2.2). In an electric current, damaged DNA loops extend towards the anode, giving the appearance of a comet with a bright head and a tail, whereas undamaged DNA remains round with no tail. The percentage of DNA in the tail (expressed as a percentage of the total DNA) and OTM (olive tail moment) values were calculated to evaluate the level of DNA damage, the latter representing the percentage of tail DNA and the displacement between the centres of mass in the head and tail regions [24]. The mean value of the percentage of tail DNA and OTM values from at least 100 nuclei/slide was taken as an index of DNA damage.

Statistical analysis
Unless stated otherwise, data are expressed as means ± S.D. All of the analyses were conducted using SPSS. Distributions of the quantitative variables were subjected to normality testing and, if necessary, log transformation was used to obtain a normal distribution. Comparisons between groups were tested by ANOVA, followed by Student–Newman–Keuls multiple range test. Distributions of categorical variables in the different groups were compared using χ² tests. Relationships between continuous variables were assessed using Pearson’s correlation coefficient analysed by the bivariate correlations method. A probability of P < 0.05 was considered significantly different.

RESULTS

Clinical characteristics of the study population
Detailed clinical characteristics of the study population are shown in Table 1, except for the information about dietary habits, tobacco consumption, physical activity and history of hyperlipidaemia and hypertension, which were comparable in the three groups. No differences were observed in anthropometric parameters, including gender distribution, age, and systolic and diastolic BP, among the groups, except for BMI, which was significantly higher in patients with IGR and diabetes compared with subjects with NGT. With regard to biochemical indices, patients with IGT had slight, but significant,
hyperglycaemia and apparent hyperinsulinaemia, thus leading to a higher HOMA-IR compared with subjects with NGT, whereas HOMA-β-cell and HbA1c remained within the normal levels. Patients with diabetes had more pronounced hyperglycaemia than the IGR group, and displayed strikingly increased HbA1c and HOMA-IR, accompanied by decreased HOMA-β-cell, when compared with both the NGT and IGR groups. Furthermore, patients with diabetes had a slightly abnormal lipid profile, with a higher triacylglycerol level than both the NGT and IGR groups, although a comparable total cholesterol level compared with the other two groups.

**Oxidative stress and antioxidative status in the study groups**

When compared with the NGT and IGR groups, patients with diabetes had a significant (P < 0.05) increase in plasma MDA (22.52 ± 6.63, 15.15 ± 4.24 and 12.21 ± 3.71 nmol/ml in patients with diabetes, patients with IGR and subjects with NGT respectively) and a significant (P < 0.05) decrease in plasma TAC (11.56 ± 2.77, 13.91 ± 3.02 and 14.02 ± 3.10 units/ml in patients with diabetes, patients with IGR and subjects with NGT respectively). Erythrocyte GSH content of patients with IGR and diabetes were within the normal reference range (78.12 ± 17.58, 78.89 ± 23.00 and 73.33 ± 20.88 mg/g of haemoglobin in patients with diabetes, patients with IGR and subjects with NGT respectively; P > 0.05), whereas these two groups exhibited significantly (P < 0.05) lower erythrocyte SOD activity than the NGT group (30.54 ± 7.39, 31.91 ± 10.45 and 36.86 ± 8.16 units/mg of haemoglobin in patients with diabetes, patients with IGR and subjects with NGT respectively).

To elucidate further the crucial role of oxidative stress in the pathogenesis of Type 2 diabetes, correlation analysis was conducted in our study population between oxidative stress, antioxidative status and IR, as well as β-cell function. Individual HOMA-IR correlated positively with BMI (r = 0.336, P < 0.001) and plasma MDA concentration (r = 0.175, P = 0.003), and correlated negatively with plasma TAC (r = −0.0129, P = 0.032), and erythrocyte SOD activity (r = −0.145, P = 0.017) and erythrocyte GSH content and HOMA-β-cell (r = 0.201, P = 0.001), and a significant inverse correlation between erythrocyte GSH content and HOMA-β-cell (r = −0.129, P = 0.032).

**DNA oxidative damage in the study population**

The comet assay, considered as a sensitive and reliable measure of DNA-strand breaks associated with incomplete excision repair and alkali-labile sites, was performed on samples from a randomly selected subset of the study population. Table 2 shows the general parameters, oxidative stress and antioxidative status of these subjects. The subset had similar values and changes to those of the whole study population, and could thus be considered as being representative of the whole study population.

Subjects with NGT had lower levels of both the percentage of tail DNA and OTM values compared with patients with IGR and diabetes, with the latter appearing to be more severe than the former (Table 2), suggesting oxidative DNA damage in patients with IGR and diabetes.

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**Table 1** Baseline characteristics of the study population

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>NGT</th>
<th>IGR</th>
<th>NDD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female (n)</td>
<td>92 (48/44)</td>
<td>78 (43/35)</td>
<td>113 (64/51)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>50.1 ± 9.6</td>
<td>51.5 ± 10.1</td>
<td>52.4 ± 10.7</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.43 ± 2.93</td>
<td>25.00 ± 3.37</td>
<td>24.71 ± 3.13</td>
</tr>
<tr>
<td>FPG (mmol/l)</td>
<td>5.14 ± 0.18</td>
<td>5.02 ± 0.10</td>
<td>5.48 ± 0.10</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>1.41 ± 0.09</td>
<td>9.69 ± 5.08</td>
<td>9.57 ± 5.91</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.50 ± 0.09</td>
<td>2.63 ± 1.43</td>
<td>4.13 ± 3.03†</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.00 ± 0.46</td>
<td>5.47 ± 0.57</td>
<td>7.73 ± 2.19†</td>
</tr>
<tr>
<td>HOMA-β-cell</td>
<td>82.52 ± 64.64</td>
<td>73.16 ± 35.83</td>
<td>37.81 ± 27.48†</td>
</tr>
<tr>
<td>Triaclyglycerol (mmol/l)</td>
<td>1.41 ± 0.90</td>
<td>1.43 ± 0.93</td>
<td>1.98 ± 1.39†</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.02 ± 1.15</td>
<td>4.81 ± 1.83</td>
<td>5.16 ± 1.90</td>
</tr>
</tbody>
</table>

**Table 2** General parameters, oxidative stress, antioxidative status and DNA oxidative damage in the subset of subjects whose plasma samples were used in the comet assay

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>NGT</th>
<th>IGR</th>
<th>NDD</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>27</td>
<td>16</td>
<td>34</td>
</tr>
<tr>
<td>Male/female (n)</td>
<td>15/12</td>
<td>8/8</td>
<td>16/18</td>
</tr>
<tr>
<td>Age (years)</td>
<td>49.5 ± 6.9</td>
<td>50.8 ± 14.2</td>
<td>53.0 ± 11.1</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.66 ± 1.87</td>
<td>25.59 ± 1.82</td>
<td>25.31 ± 1.90†</td>
</tr>
<tr>
<td>FPG (mmol/l)</td>
<td>5.04 ± 0.39</td>
<td>5.79 ± 0.30</td>
<td>9.18 ± 2.58†</td>
</tr>
<tr>
<td>FPI (m-units/l)</td>
<td>6.37 ± 2.36</td>
<td>10.45 ± 7.66</td>
<td>9.94 ± 5.48</td>
</tr>
<tr>
<td>HOMA IR</td>
<td>1.42 ± 0.51</td>
<td>2.66 ± 1.86†</td>
<td>3.45 ± 1.84†</td>
</tr>
<tr>
<td>HOMA-β-cell</td>
<td>88.43 ± 42.56</td>
<td>86.13 ± 53.55</td>
<td>40.76 ± 34.60†</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>4.93 ± 0.52</td>
<td>5.60 ± 0.78</td>
<td>8.01 ± 1.89†</td>
</tr>
<tr>
<td>Triaclyglycerol (mmol/l)</td>
<td>1.75 ± 1.17</td>
<td>2.09 ± 0.95</td>
<td>2.14 ± 1.21</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.95 ± 0.60</td>
<td>5.42 ± 0.73</td>
<td>4.93 ± 0.83</td>
</tr>
<tr>
<td>MDA (nmol/ml)</td>
<td>10.77 ± 5.59</td>
<td>15.22 ± 5.57</td>
<td>19.13 ± 7.11†</td>
</tr>
<tr>
<td>TAC (units/ml)</td>
<td>15.08 ± 3.31</td>
<td>14.00 ± 4.16</td>
<td>11.07 ± 4.42†</td>
</tr>
<tr>
<td>Tail DNA (%)</td>
<td>3.76 ± 1.19</td>
<td>7.15 ± 1.58†</td>
<td>9.95 ± 2.48†</td>
</tr>
<tr>
<td>OTM value</td>
<td>0.87 ± 0.23</td>
<td>2.06 ± 0.46†</td>
<td>3.09 ± 0.75†</td>
</tr>
</tbody>
</table>

*P < 0.05 compared with the NGT group; †P < 0.05 compared with the IGR group; ‡P < 0.05 compared with the NGT group.
Linear correlation analysis of the percentage of tail DNA showed significant positive correlations with BMI \((r = 0.517, P = 0.001)\) and HOMA-IR \((r = 0.349, P = 0.025)\), and a negative association with HOMA-\(\beta\)-cell \((r = -0.45, P = 0.003)\). Similarly, the OTM value correlated directly with FPG \((r = 0.566, P < 0.001)\) and HOMA-IR \((r = 0.324, P = 0.039)\), and was inversely correlated with HOMA-\(\beta\)-cell \((r = -0.498, P = 0.001)\). No significant associations were observed between the percentage of tail DNA and the OTM value with either plasma MDA concentration or TAC level, although the \(P\) values did approach statistical significance (results not shown).

**DISCUSSION**

In the present study, we found that patients with IGR had evident IR (associated with a high BMI value) but normal \(\beta\)-cell function and, thus, they could compensate for insulin insensitivity by increasing insulin secretion and displaying slight hyperglycaemia, which was just above the value of normoglycaemia, as well as having a normal HbA1c and lipid profile. As expected, IR was aggravated in patients with Type 2 diabetes. Although insulin secretion increased significantly compared with subjects with NGT, typical characteristics of Type 2 diabetes, such as apparent hyperglycaemia, increased HbA1c, and an abnormal lipid profile, still occurred, which are attributed to significant defects in \(\beta\)-cell secretion, confirming further the concept that IR alone is not sufficient to induce Type 2 diabetes in the absence of \(\beta\)-cell defects [4–6].

It is widely postulated that the aetiology of Type 2 diabetes has a strong association with oxidative stress, originating from increased free radical production and decreased antioxidant potential [25]. However, a number of studies on the role of oxidative stress in Type 2 diabetes have shown inconsistent results [26], and this discrepancy may exist with respect to patient age, duration of diabetes, treatment methods, techniques used to measure oxidative stress etc. Even so, the most common results reported in patients with Type 2 diabetes are increased oxidative stress and impaired antioxidative defence systems [13,26,27]. However, data on oxidative stress and oxidative damage in patients with IGR and patients with newly diagnosed Type 2 diabetes are scarce and, thus, in the present study, we investigated the oxidative stress status and oxidative DNA damage in these subjects by evaluating a number of the most widely used plasma and intracellular biomarkers, including plasma MDA levels (oxidative stress), erythrocyte GSH content and SOD activity, plasma TAC level (antioxidative defence system) and the comet assay (oxidative DNA damage).

Compared with subjects with NGT, no differences were observed in oxidative stress and antioxidant markers in patients with IGR, except for a decrease in erythrocyte SOD activity and a tendency towards an increase in MDA levels. This decrease was possibly correlated with slight hyperglycaemia-induced oxidative stress, which is promoted by the overproduction of free radicals arising from hyperglycaemia-induced glucose auto-oxidation, non-enzymatic glycation of proteins and increased sorbitol pathway [7,12]. Thus erythrocyte SOD activity appeared to be a sensitive and early marker of oxidative stress in IGR. In agreement with many previous studies [7,26–28], we have found increased accumulation of lipid peroxidation end products (plasma MDA levels) in patients with newly diagnosed diabetes caused by hyperglycaemia. Concomitantly, decreased erythrocyte SOD activity was observed in patients with Type 2 diabetes, which was possibly associated with the progressive glycation of this enzymatic protein caused by high HbA1c levels in these patients [26,29–31].

Approx. 50% of erythrocyte SOD activity in patients with Type 2 diabetes has been reported to be glycated, resulting in the decreased activity [32]. Although erythrocyte GSH content in patients with diabetes was not different compared with subjects with NGT, this finding is inconsistent with previous studies reporting a decrease in erythrocyte GSH concentration [26,27,33], suggesting a potential problem of using a specific antioxidant compound to evaluate oxidative stress states. Thus, in accordance with previous studies [27,34], we used the plasma TAC level as an indicator of antioxidant status, and observed a depletion in plasma TAC in patients with Type 2 diabetes.

Subsequently, we investigated the inter-relationship between oxidative stress status and IR and pancreatic \(\beta\)-cell function. We found that there was a significant positive association with MDA level, but a negative association with TAC level and SOD activity. In contrast, pancreatic \(\beta\)-cell function was inversely correlated with the GSH level, although positively correlated with the TAC status. These conclusions were supported by our results of these biomarkers in a population with a different stage of abnormal glucose metabolism (IFG), and suggested the involvement of oxidative stress induced by ‘glucose toxicity’ in the progression and pathogenesis of Type 2 diabetes.

To elucidate further the underlying mechanism mediating the oxidative-stress-induced outcome in Type 2 diabetes, especially \(\beta\)-cell dysfunction, we subsequently evaluated oxidative DNA damage in PBMCs. Oxidative DNA damage is usually evaluated using a comet assay in white blood cells [35–38] or by measuring the oxidized base 8-OHdG (8-hydroxy-2-deoxyguanosine) in white blood cells or urine [39–41]. In the present study, we selected samples from a subset of our study population and performed comet assays as a measure of DNA-strand-break damage, as the technique is less susceptible to artefacts than measuring 8-OHdG [42,43].
from the comet assay demonstrated that patients with newly diagnosed diabetes had apparent DNA damage, with significantly higher percentages of tail DNA and OTM values as well as typical comet-like nuclei under photomicrograph examination, indicative of increased DNA damage in blood cells from patients with diabetes [37–39,44]. However, several studies have shown a lack of association between diabetes and increased DNA damage level [45,46], possibly due to discrepancies in glycaemic control, duration of diabetes and the type of cells used in the comet assay [44]. Similar, but less significant, DNA damage was also observed in patients with IGR, concurrent with the slightly higher oxidative stress status. Furthermore, we also found a significant correlation between DNA damage and hyperglycaemia, as reported previously [37,39]. Oxidative DNA damage appeared to play an important role in the development of pancreatic β-cell dysfunction and IR, as demonstrated by the strong positive and inverse correlations between DNA damage and HOMA-IR and HOMA-β-cell.

The present study could depict the possible underlying mechanisms of the pathogenesis of Type 2 diabetes by investigating oxidative stress pathways and DNA damage in IGR and newly diagnosed Type 2 diabetes. Even in the IGR state, the slightly increased FPG or glucose load after an OGTT resulted in increased oxidative stress or compromised antioxidative defence systems or both, leading to oxidative DNA damage, which possibly contributed to pancreatic β-cell dysfunction, IR and more pronounced hyperglycaemia. This vicious circle is proposed to finally induce the deterioration to diabetes. These results not only confirm the important role of oxidative stress in the aetiology of Type 2 diabetes, but also have important implications for the prevention of Type 2 diabetes in pre-diabetic patients (patients with IGR).

However, we cannot draw a definite conclusion that DNA damage resulting from reduced antioxidative defences is the cause of the progression of diabetes as more direct evidence is required. Furthermore, long-term prospective clinical investigations are required to develop predictive biomarker profiles to identify individuals with IGR at particularly high risk of developing Type 2 diabetes.

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