Original article

DNA damage and plasma antioxidant indices in Bangladeshi type 2 diabetic patients

M. Arif a,*, M.R. Islam a, T.M.Z. Waise a, F. Hassan a, S.I. Mondal b, Y. Kabir c

a Department of Biochemistry and Molecular Biology, University of Dhaka, Dhaka 1000, Bangladesh
b Department of Genetics, Shahjalal University of Science and Technology, Sylhet, Bangladesh
c Department of Family Sciences, College for Women, Kuwait University, Kuwait

Received 13 February 2009; received in revised form 21 May 2009; accepted 26 May 2009
Available online 29 December 2009

Abstract

Background and aim. – Diabetes mellitus is a complex metabolic disorder characterized by a disturbance in glucose metabolism. Recent evidence suggests that increased oxidative stress as well as alteration of antioxidant capacity may be related to the complications seen in patients with type 2 diabetes. The aim of this study was to measure serum antioxidant status in type 2 diabetic patients and to assess its relationship with oxidative DNA damage.

Methods. – A total of 57 subjects were included in this study. Of these, 32 were type 2 diabetic patients and 25 were non-diabetic subjects. Comet assay was used to quantify the level of DNA damage in lymphocytes. Spectrophotometric methods were used to assess serum levels of malondialdehyde (MDA) and protein carbonyl, and serum activity of superoxide dismutase (SOD) and the protein thiol (P-SH) group.

Results. – A significant increase in mean comet tail DNA, indicating DNA damage, was observed in diabetic patients compared with controls. Diabetic patients had significantly higher levels of MDA and protein carbonyl in parallel with significant decreases in levels of SOD and the P-SH group compared with controls. Serum SOD was also inversely correlated with the increase in comet tail DNA.

Conclusion. – These results indicate the presence of significant lipid peroxidation, protein oxidation and oxidative DNA damage in patients with diabetes. Perturbation of glucose homoeostasis was associated with an increase in oxidants and a concomitant decrease of antioxidant enzymes in the type 2 diabetic patients’ blood. The present study suggests that the status of oxidant–antioxidant imbalance may be one of the mechanisms leading to the DNA damage detected in the lymphocytes of type 2 diabetic patients.

© 2009 Elsevier Masson SAS. All rights reserved.

Keywords: DNA; Comet assay; Malondialdehyde; Protein carbonyl; Superoxide dismutase; Protein thiol; Type 2 diabetes mellitus; Oxidative stress

Résumé

Altérations de l’ADN et marqueurs plasmatiques du stress oxydant chez des diabétiques de type 2 du Bangladesh.

Objectifs. – Le diabète sucré est un trouble métabolique complexe caractérisé par des anomalies du métabolisme du glucose. Des données récentes suggèrent qu’une augmentation du stress oxydant associée à une diminution des capacités anti-oxydantes pourrait être impliquée dans le développement des complications du diabète de type 2 (DT2). L’objectif de cette étude était d’évaluer le statut anti-oxydant plasmatique de patients atteints de (DT2) et de rechercher ses relations avec les altérations de l’ADN.

Méthodes. – Cinquante sept sujets ont été inclus dans cette étude, 32 DT2 et 24 témoins. La méthode Comet a été utilisée pour quantifier les altérations de l’ADN lymphocytaire. Les concentrations sériques de di-aldéhyde malonique (MDA), les protéines carbonylées, l’activité de la superoxyde dismutase (SOD) et des protéines du groupe thiol (-SH P) ont été mesurés par spectrophotométrie.

Résultats. – Une augmentation significative des altérations de l’ADN par la méthode Comet a été observées chez les patients atteints de DT2 comparés aux témoins. Les patients diabétiques avaient des concentrations de MDA élevées, parallèlement à celles des protéines carbonylées et une diminution importante de l’activité SOD et du groupe P-SH par rapport aux témoins. L’activité sérique de la SOD était en corrélation inverse avec les altérations de l’ADN.

* Corresponding author.
E-mail address: mari567@yahoo.com (M. Arif).

1262-3636/$ – see front matter © 2009 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.diabet.2009.05.007
1. Introduction

Diabetes mellitus may result in an increased production of reactive oxygen species (ROS) via numerous physiological pathways. Small amounts of ROS, including hydroxyl radicals ("OH), superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2), are constantly being generated in aerobic organisms in response to both external and internal stimuli. They are physiologically important for health, but may be detrimental if present in excess quantities [1,2]. Oxidative stress, defined as the loss of balance between ROS production and antioxidant defenses [3,4], leads to eventual macromolecular damage. As lipid peroxidation and protein carbonyl group contents are used as indicators of oxidative stress, their evaluation in plasma serves as a marker of free radical activity [5]. Oxidative degradation of polyunsaturated fatty acids, which forms malondialdehyde (MDA) and is usually measured as thiobarbituric acid-reactive substances (TBARS), or lipid peroxides, is the most well-studied, biologically relevant, free radical reaction [6]. Protein thiol (P-SH) groups and reduced glutathione (GSH) are particularly important as they prevent oxidative damage and serve as potent intracellular antioxidants [7]. P-SH can, in turn, be converted to non-toxic compounds via catalase (CAT) and (selenium-containing) glutathione peroxidase [8]. Therefore, the measurement of protein sulphydryl groups and protein carbonyl content might be practical ways to assess antioxidant status. In addition, superoxide dismutase (SOD) is thought to play a major role in the first line of antioxidant defense by catalyzing the dismutation of O_2^- to molecular oxygen (O_2) and H_2O_2, which can, in turn, be converted to non-toxic compounds via catalase (CAT) and (selenium-containing) glutathione peroxidase [9].

Increasing evidence suggests that oxygen free radicals induce a variety of lesions in DNA, including oxidized bases, DNA strand breaks and DNA–protein crosslink formation [10]. In addition, it has been shown that hydroxyl radicals produced by the Fenton reaction in the presence of transition metal ions are also responsible for DNA damage [11]. Single-cell gel electrophoresis—also known as ‘comet assay’ because of the characteristic resultant comet-like cell appearance—is a simple, sensitive and rapid method effective for detecting DNA damage. It can be used to estimate DNA damage at the individual cell level through strand breaks, open repair sites, crosslinks and alkali labile sites generated from oxidative stress [12,13,14]. Although there have been published reports of oxidative stress and antioxidant status in type 2 diabetic patients in relation to some diseases [15,16], in the present study, our objective was to determine the association between oxidant–antioxidant contents and increased DNA damage in type 2 diabetic patients in the Bangladeshi population.

2. Materials and methods

2.1. Study subjects

A total of 57 subjects—including 32 type 2 diabetic patients—attending the outpatients department of the central hospital (BIRDEM) of the Diabetic Association of Bangladesh (DAB) were recruited on the basis of their glycosylated haemoglobin (HbA1c) and fasting blood glucose levels (Table 1). Patients with other systemic illnesses and those receiving antioxidants and/or drugs were excluded. In addition, 25 non-diabetic, age- and gender-matched subjects were also included as controls.

2.2. Sample collection

Blood samples were collected in EDTA-containing tubes after overnight (10–12 h) fasting. Plasma was separated, and used for glucose (autoanalyzer; Beckman, USA) and other biochemical assays. Lymphocytes, isolated with the use of Histopaque, were used in the comet assay, and HbA1c was analyzed by high-performance liquid chromatography (HPLC).

2.3. Comet assay

The comet assay was carried out according to Tice et al. [13], but with slight modification. Lymphocytes were suspended in 0.7% low-melting-point agarose in phosphate buffered saline (PBS) at 37 °C and placed on microscopic slides with a layer of 1% agarose. The slides were immersed in lysis solution at 4 °C for 1 h, and followed by electrophoresis at 25 V, 300 mA, for 40 min at steady temperature. The slides

### Table 1
Clinical characteristics of non-diabetic subjects and type 2 diabetic patients.

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 25)</th>
<th>T2DM patients (n = 32)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male/female)</td>
<td>15/10</td>
<td>18/14</td>
<td>ns</td>
</tr>
<tr>
<td>Age (year)</td>
<td>48.83 ± 8.3</td>
<td>47.25 ± 4.60</td>
<td>ns</td>
</tr>
<tr>
<td>Body mass index (BMI)</td>
<td>28.30 ± 2.4</td>
<td>29.2 ± 2.60</td>
<td>ns</td>
</tr>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>90.0 ± 9.5</td>
<td>146.0 ± 6.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>2.92 ± 0.64</td>
<td>8.10 ± 2.88</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Duration of diabetes (yr)</td>
<td>NA</td>
<td>5.60 ± 1.81</td>
<td>−</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SD (standard deviation); T2DM: type 2 diabetes mellitus; ns: not significant; NA: not applicable.
were then silver-stained, as described by Nadin et al. [17]. All steps, from sample collection to electrophoresis, were conducted under yellow light to minimize the possibility of cellular DNA damage. The slides were then analyzed microscopically with the use of Comet Assay Software Project (CASP, version 1.2.2) software to determine the following comet parameters: percentage of DNA in the comet tail; and tail moment.

2.4. Lipid-peroxidation assay

Plasma lipid peroxidation was determined as described by Ohkawa et al. [18]. Plasma proteins were precipitated in trichloroacetic acid (TCA). MDA, produced during lipid peroxidation, reacts with thiobarbituric acid (TBA) and generates a pink-colored complex. After some further steps, the absorbance of the supernatant was measured spectrophotometrically at 532 nm using 1,1,3,3-tetraethoxypropane as a standard. TBARS were calculated as nmol/mg protein.

2.5. Estimation of protein carbonyl content

The protein carbonyl content of plasma was estimated as performed in Chakrabarti et al. [19], and the results presented as nmol/mg protein.

2.6. Estimation of protein sulphydryl content

Plasma sulphhydryl levels were determined using 5,5'-dithiobis-2-nitrobenzoic acid (Ellman’s reagent), as described previously by Habeeb et al. [20].

2.7. Superoxide dismutase assay

SOD assay was performed with slight modification of the method described by Beauchamp et al. [21]. The reaction mixture contained 1.1 mL of 59-mM phosphate buffer (pH 7.4), 75 μL of 20-mM methionine, 40 μL of 1% Triton X-100, 75 μL of 10-mM HAC (hydroxyl amine hydrochloride), 100 mL of 78-μM EDTA and 100 μL of plasma. The reaction was started by the addition of 80 μL of freshly prepared 40-μM riboflavin, and the tubes placed under light for 10 min. Then, 1 mL of Griess reagent was added to each tube, and the optical density read at 543 nm after 10 min and compared with the controls, which were kept under dark conditions. The results were expressed as nmol SOD/mg protein.

2.8. Statistical analysis

For the statistical analyses, the unpaired t test was used. Spearman’s rank correlation coefficient was used to evaluate relationship variables, and linear regression analysis was used to examine the relationship between two variables. A value of \( P < 0.05 \) was considered statistically significant.

3. Results

3.1. Fasting glucose and glycosylated hemoglobin

The clinical characteristics of the study groups are presented in Table 1. Participants were well matched for age and gender, and there were no significant differences in age and body mass index (BMI) across the groups. However, the type 2 diabetic patients—both men and women—had significantly higher levels of fasting blood glucose (Table 1), whereas the controls had normal levels with no diabetic complications. In addition, the relative HbA1c levels were significantly higher in the diabetic patients versus the controls (8.1 ± 2.88% vs 2.92 ± 0.64%, respectively), which is in accordance with the findings of Chan et al. [22]. These results indicate the poor blood glucose control in type 2 diabetes patients.

3.2. Antioxidant content

The antioxidant status between the two study groups was assessed by measuring SOD and protein thiols. In diabetic patients, SOD activity was significantly lower (5.54 ± 0.76) compared with that in the controls (8.20 ± 0.59; \( P < 0.05 \); Fig. 1A). The mean difference in P-SH levels between the diabetic patients and non-diabetic controls was also significant (12.43 ± 1.54 vs 18.24 ± 1.12, respectively; \( P < 0.05 \); Fig. 1B). Furthermore, there was an observed inverse correlation between tail moment and antioxidant content \( r = -0.77 \) (\( P < 0.05 \)) for SOD; \( r = -0.63 \) for protein thiols.

3.3. Prooxidant status

Lipid peroxidation and protein carbonyl group content were determined to assess the extent of oxidative stress. The lipid peroxidation rate was measured by determining TBARS in the subjects’ plasma. Levels of lipid peroxides (TBARS) significantly increased in diabetic patients compared with controls (91.96 ± 7.82 vs 61.90 ± 4.90, respectively; \( P < 0.01 \); Fig. 1C). The change in protein carbonyl content between the diabetics and non-diabetic controls (Fig. 1D) was also statistically significant (51.74 ± 4.33 vs 30.20 ± 4.55, respectively; \( P < 0.05 \)). Moreover, levels of MDA (\( r = 0.70 \); \( P < 0.05 \)) and protein carbonyl content (\( r = 0.82 \); \( P < 0.05 \)) were both positively correlated with tail moment.

3.4. DNA damage

Comet images of blood cells from the diabetic patients showed abnormal appearances (long tail nucleus; Fig. 2B) whereas, in the control group, there was no such evidence of comet formation (Fig. 2A). The extent of DNA damage can be expressed by measuring the percent of DNA present in the tail region by comet analysis. In the type 2 diabetic patients, tail DNA increased to up to 64% compared with only 28% in the controls (Fig. 2C). A commonly used marker of DNA damage is the tail moment (percent of tail DNA × tail length), which clearly showed significant (\( P < 0.01 \)) DNA damage in the dia-
Fig. 1. Antioxidant and prooxidant status in the controls and type 2 diabetes (DM) patients: levels of SOD (A) and protein thiol (B) were measured in the non-diabetic controls and type 2 DM patients. Changes in the levels of TBARS (C) and protein carbonyl content (D) were calculated in both study groups. Data are expressed as means ± SEM. *P < 0.05; **P < 0.01.

4. Discussion

There is growing evidence that type 2 diabetes is associated with severe lipid peroxidation, and oxidative damage of DNA and proteins [23]. In the present study, we measured the plasma antioxidant status in type 2 diabetic patients from the Bangladeshi population and examined its relationship to DNA damage.

On evaluating the subjects’ antioxidant status, we found reduced SOD activity in the diabetic patients (Fig. 1A) compared with non-diabetic subjects, which is consistent with the findings of other studies of SOD [24,25], and do not corroborate those of a study [26] that found no significant change in SOD activity in type 2 diabetics. In patients with type 2 diabetes, auto-oxidation of glucose leads to hydrogen peroxide formation that, in turn, inactivates SOD [27]. This suggests that the accumulation of hydrogen peroxide may be one explanation for the decreased SOD activity seen in such patients. The primary catalytic cellular defense mechanism that protects cells and tissues against the potentially destructive reactions of superoxide radicals and their derivatives is copper–zinc SOD (Cu/Zn SOD). It has been observed that SOD can be rapidly induced under certain conditions, such as when cells or organisms are exposed to oxidative stress [27]. The highest SOD activity in red blood cells has been reported at the onset of diabetes, followed by a subsequent decrease in activity [28]. In the present study, the higher the level of glycosylated haemoglobin, the more prolonged was the diabetic condition (Table 1). Therefore, low SOD activity in type 2 diabetes could be due to the longer disease duration, with SOD induced at an early stage, after which its activity progressively decreases. Plasma protein thiols are considered to be important physiological free radical scavengers and serve as potent antioxidants through a number of mechanisms. Protein thiols can preemptively scavenge the oxidants that initiate peroxidation and, thus, avoid oxidative damage. In the present study, protein thiol contents were found to be significantly reduced in type 2 diabetics compared with the non-diabetic controls (Fig. 1B). This suggests that our results hold up reasonably well against other findings that propose decreased protein thiol levels in type 2 diabetic patients [29].

Oxidative stress leads to the increased production of oxygen free radicals such as superoxides (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxide (OH$^-$) radicals that weaken the body’s
antioxidant defense mechanisms. Oxidants are highly reactive compounds with a very short half-life, and their determination in vivo is generally not feasible. In contrast, lipids, proteins, carbohydrates and DNA after modification by oxyradicals have a longer natural life that is easily measured by biochemical assays. To evaluate oxidative stress, many biomarkers have been developed, including lipid peroxidation products (MDA), protein oxidation products (protein carbonyl groups) and damaged DNA products. Several authors have reported elevated levels of lipid peroxides in diabetic patients [30,31], while others could find no significant change in lipid peroxidation in diabetics [32]. TBARS are used as an index of lipid peroxidation and oxidative stress. In the present study, levels of TBARS were significantly increased in the plasma of all diabetic patients compared with the controls (Fig. 1C).

These data clearly reveal the effects of prolonged diabetes on lipid peroxidation and oxidative stress in the Bangladeshi population. This has led us to propose that glucose undergoes oxidation with higher nicotinamide adenine dinucleotide phosphatase (NADPH) production that, in turn, speeds up peroxidation, similar to that of lipids and other substances in poorly controlled diabetes. ROS-mediated reactions lead to the formation of protein carbonyl derivatives that serve as markers of ROS-mediated protein damage. Elevated protein oxidation has often been associated with diseases such as Alzheimer’s, diabetes, ageing and cancer [33], and several studies have shown increased protein carbonyl contents in patients with diabetes [34,35]. In line with such previous findings, significantly increased levels of protein carbonyl were observed in our type 2 diabetics (Fig. 1D). Increased ROS may also interact with proteins, rendering them vulnerable to oxidative modification and, eventually, degradation.

Oxidative stress not only damages cellular proteins, but can also significantly affect DNA and generate various base modifications. Our comet analysis showed that the control group had compact DNA and maintained the circular form of a normal nucleus, with no evidence of comet formation (Fig. 2A). In contrast, cells from type 2 diabetic patients exhibited a distorted appearance, indicating substantial DNA damage (Fig. 2B) that may have been due to oxidative stress, according to our oxidative stress data and those of another study [36], which revealed that patients with type 2 diabetes had higher oxidative DNA damage than did non-diabetic subjects. Furthermore, we found significant positive correlations between fasting blood glucose and tail DNA percentages (Fig. 3A) and tail moments (Fig. 3B). Indeed, our data confirm the hypothesis that DNA damage may be among the more important factors for the onset and/or further aggravation of diabetic complications in poorly controlled diabetes [25]. Tail moments were also significantly and positively correlated with TBARS and protein carbonyl group content, and inversely correlated with SOD activity and protein thiol content. Thus, the present study findings suggest that the status of oxidant–antioxidant imbalance could be one of the mechanisms leading to the DNA damage detected in lymphocytes of type 2 diabetic patients. Nevertheless, the dietary habits of such patients cannot be dismissed, as some studies have shown that...

Fig. 2. Comet images of blood cells from diabetic patients: the extent of DNA damage was assessed by comet assay, coupled with silver staining, using lymphocytes from the controls (A) and type 2 DM patients (B). Quantitative analysis of comet images shows tail DNA (%) (C) and tail moment (D). Data are expressed as means ± SEM. *P < 0.05; **P < 0.01 (Bonferroni test).
the presence of high ascorbic acid levels reduces DNA damage to the same degree as it reduces hyperglycaemia [37].

In conclusion, the present study demonstrates that elevated levels of oxidative DNA damage are seen in the blood cells of type 2 diabetic patients compared with age-matched non-diabetic subjects in the Bangladeshi population. Such oxidative DNA damage is closely associated with a significant reduction in the patient’s antioxidant contents and with upregulation of prooxidant-induced DNA damage, suggesting decreased efficacy of DNA repair mechanisms and the possibility of cancer onset.

Conflict of interest

No conflict of interest relevant to this article was reported.

References


