SUMMARY

Diabetes is associated with excessive production of reactive oxygen species (ROS), which can damage cellular macromolecules. The aim of the study was to detect oxidative DNA damage in type 2 diabetic patients using the comet assay, and to study the relationship between DNA damage and hyperglycemia lipid profile in diabetic patients. The study population consisted of 28 patients with type 2 diabetes mellitus and 25 healthy volunteers, age and sex matched with the patients, as controls. Lipid profile, fasting blood glucose and glycosylated hemoglobin (HbA1c) were assessed in patients and controls. Comet assay was used to detect DNA damage. The percent of DNA damage of peripheral blood mononuclear cells was higher in diabetic patients (32.6±25.2) compared to healthy controls (3.7±1.3) (p<0.001). Pearson correlation analysis showed a significant positive correlation of DNA damage with fasting blood glucose and glycated hemoglobin, but not with serum total cholesterol, triglycerides, HDL-c and LDL-c. In conclusion, type 2 diabetic patients have more oxidative DNA damage than normal controls and poor glycemic control may aggravate this damage. Dyslipidemia is not a contributing factor for DNA damage in diabetes.

INTRODUCTION

Diabetes mellitus is a chronic metabolic syndrome. It is one of the most challenging health problems in the 21st century. The prevalence of diabetes is increasing globally, the number of diabetics is expected to increase to 366 million by 2030 (1). Patients with type 2 diabetes suffer from several complications such as atherosclerosis, retinopathy, neuropathy and nephropathy with devastating effect on morbidity and mortality (2).
There is considerable evidence that hyperglycemia results in the generation of reactive oxygen species (ROS), ultimately leading to increased oxidative stress in a variety of tissues, and playing an important role in diabetic complications (3,4). Oxidative stress leads to protein, lipid, and DNA modifications that cause cellular dysfunction and this could have teratogenic or carcinogenic consequences (5).

During the past ten years, there has been increasing awareness of the effect of DNA damage in chronic diseases. Single cell gel electrophoresis (SCGE) or comet assay to measure DNA damage was first developed by Östling and Johansson in 1984 (6). It is a sensitive, simple, inexpensive, and rapid method that can be used to detect DNA damage to individual cells and reveal the presence of double-strand breaks, single-strand breaks and alkali-labile sites (7). It has been widely used in studies on DNA repair, genetic toxicology, radiation, pollution and ageing (8,9).

The aim of this study was to detect the oxidative DNA damage in patients with type 2 diabetes mellitus and to investigate the relation between oxidative DNA damage and hyperglycemia and lipid profile.

MATERIALS AND METHODS

Twenty-eight patients with type 2 diabetes mellitus were recruited from the outpatient clinic of Medical Services Unit at the National Research Center. There were 8 men and 20 women, age range from 45 to 61 years, mean age 52.5±5.3 years. Twenty-five healthy volunteers, 10 men and 15 women, age range from 45 to 60 years, mean age 53.1±6.7 years, served as control group. We excluded any patient with a history of smoking, acute or chronic infections, coronary artery disease, congestive heart failure, chronic liver disease, diabetic nephropathy, rheumatic disease, cancer, and patients who had recently undergone radiological procedures (1 month previously). None of the patients was taking lipid lowering drugs.

Participants were subjected to detailed history for collection of demographic data and recording of relevant medical history and medications. Thorough clinical examination and height and weight measurement for calculation of body mass index (BMI) were also done in patients and controls. A written informed consent was obtained from each participant and the study was approved by the Ethics Committee.

Laboratory methods

Five milliliters of venous blood was withdrawn from both healthy individuals and patients fasting for 10 hours into two sterile vacutainers, one containing EDTA and the other without additives to separate serum.

Serum samples were assayed within 2 hours for fasting blood glucose, glycosylated hemoglobin HbA1c and lipid profile: total cholesterol, triglycerides and high-density lipoprotein cholesterol (HDL-c) using the Olympus AU 400 automated clinical chemistry analyzer. Low-density lipoprotein cholesterol (LDL-c) was calculated by Friedewald formula (10). DNA damage was detected by the comet assay.

Measurement of comet assay

Cell preparation

Peripheral blood leukocytes were isolated by centrifugation (30 min at 1300 g) in Ficoll-Paque density gradient (Pharmacia LKB Biotechnology, Piscataway, NJ, USA). After centrifugation, leukocytes in the buffy coat were aspirated and washed twice by phosphate-buffered saline (PBS) at pH 7.4.

Preparation of cell microgels on slides

All the procedures for the alkaline comet assay were done at low temperature to minimize spontaneous DNA damage. The comet assay was performed according to Singh et al. (11) with modifications according to Blasiak et al. (12). Cell microgels were prepared as layers. The first gel layer was made by applying 100 μL of normal melting point agarose (0.7%) onto a precleaned microscope charged slides and coverslipped gently. The coverslip was removed after the agarose solidified at 4 °C. Low melting-point
agarose (0.5%) was prepared in 100 mmol/L PBS and kept at 37 °C. Approximately 1500 peripheral blood lymphocytes were mixed with the low melting-point agarose and 100 μL of the mixture was applied to the first gel layer. The slides were then covered with a coverslip and placed at 4 °C for solidification. After the second layer had solidified, the coverslips were removed from the cell microgels. A final layer of low-melting agarose was added followed by coverslips, left to solidify for 10 minutes, and then the coverslips were removed.

**Lysis of cells, DNA unwinding, gel electrophoresis, DNA staining**

The slides were covered with 100 mL of fresh lysis buffer (2.5 mol/L NaCl, 100 mmol/L EDTA, 1% sodium hydroxide, 10 mmol/L Tris, 1% Triton X-100, 10% DMSO (pH 10) at 4 °C for 1 h. After draining, microgel slides were treated with DNA unwinding solution (300 mmol/L NaOH, 1 mmol/L EDTA, pH 13) for 30 min at 4 °C, and placed directly into a horizontal gel electrophoresis chamber filled with DNA-unwinding solution. Gels were run with constant current (300 mA at 4 °C) for 30 min. After electrophoresis, the microgels were neutralized with 0.4 M Trisma base at pH 7.5 for 10 min. The slides were stained with 20 μL ethidium bromide (10 μg/mL).

**Visualization and analysis of comet slides**

The slides were examined at 400× magnification using an inverted fluorescence microscope (IX70, Olympus, Tokyo, Japan) equipped with an excitation filter of 549 nm and a barrier filter of 590 nm, attached to a video camera (Olympus). Damaged cells were visualized by the "comet appearance", with a brightly fluorescent head and a tail to one side formed by the DNA containing strand breaks that were drawn away during electrophoresis. Samples were analyzed by counting damaged cell out of 100 cells per slide to calculate the percent of damage.

**Statistical analysis**

Data are presented as mean ±SD. The compiled data were computerized and analyzed by SPSS PC+, version 14. The following tests of significance were used: t-test between means to analyze mean difference. A value of $p≤0.05$ was considered significant, $p<0.001$ was considered highly significant, and $p>0.05$ was considered nonsignificant. Relationships between continuous variables were assessed using Pearson’s correlation coefficient.

**RESULTS**

The study included 28 patients with type 2 diabetes mellitus and 25 healthy individuals as control group. The mean age of the patients was 52.5±5.3 years.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Diabetic patients (n=28)</th>
<th>Controls (n=25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs), mean±SD</td>
<td>52.5±5.3</td>
<td>53.1±6.7</td>
</tr>
<tr>
<td>Sex: male/female</td>
<td>8/20</td>
<td>10/15</td>
</tr>
<tr>
<td>BMI (kg/m²), mean±SD</td>
<td>31.7±3.7</td>
<td>30.2±1.8</td>
</tr>
<tr>
<td>Duration (yrs), mean±SD</td>
<td>9.7±5.1</td>
<td>-</td>
</tr>
<tr>
<td>Fasting blood glucose (mg/dL), mean ± SD</td>
<td>185.86±70.35*</td>
<td>86.56±6.25</td>
</tr>
<tr>
<td>HbA₁c (%), mean±SD</td>
<td>9.4±3.3*</td>
<td>6±0.7</td>
</tr>
<tr>
<td>Cholesterol (mg/dL), mean±SD</td>
<td>229.7±56.2</td>
<td>217.6±68.9</td>
</tr>
<tr>
<td>Triglycerides (mg/dL), mean±SD</td>
<td>170±88.3</td>
<td>133.5±85.3</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL), mean±SD</td>
<td>45.8±12.6*</td>
<td>62.6±21.4</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL), mean±SD</td>
<td>148±51</td>
<td>129.8±56</td>
</tr>
<tr>
<td>DNA damage (%), mean±SD</td>
<td>32.6±25.2*</td>
<td>3.7±1.3</td>
</tr>
</tbody>
</table>

*p<0.001; BMI = body mass index; HDL = high density lipoprotein; LDL = low density lipoprotein
None of the patients was taking antioxidant supplement. Demographic and laboratory data on the patients and controls are shown in Table 1.

The number of DNA strand breaks was significantly higher in diabetic patients compared to controls. The number of DNA strand breaks correlated positively with fasting blood glucose and HbA1c, while no significant correlation was found with serum total cholesterol, triglycerides, HDL-c, LDL-c and BMI (Table 2).

**DISCUSSION**

Diabetes mellitus is associated with oxidative stress, leading to protein, lipid and DNA modifications. Oxidative DNA damage is usually evaluated using the comet assay in white blood cells (13,14), or by measuring the oxidized base 8-OHdG (8-hydroxy-2-deoxy-guanosine) in white blood cells or urine (15,16).

In the present study, we used the comet assay as a measure of DNA strand-break damage, as the technique is less susceptible to artifacts than measuring 8-OHdG, and it is a sensitive, simple method to detect very low levels of damage (17).

The present study revealed an increased number of DNA strand breaks in peripheral blood leukocytes of diabetic patients compared to healthy controls. The production of ROS and lipid peroxidation are increased in diabetic patients (18). Reactive oxygen species can damage cellular macromolecules, leading to DNA and protein modification and lipid per-oxidation. The elevated ROS in diabetes can cause strand breaks in DNA and base modifications including oxidation of guanine residues to 8-OHdG, an oxidized nucleoside of DNA, which is the most frequently detected and studied DNA lesion (19).

Previous studies concerning DNA damage and diabetes revealed contradictory results. Several studies showed an increased extent of DNA damage in type 2 diabetic patients compared to controls (14,20,21). On the other hand, other studies showed the lack of association between diabetes and increased DNA damage levels (22,23). The discrepancy between different studies is, possibly, due to difference in glycemic control, duration of diabetes or the type of cell used in the comet assay (21).

Goodarzi et al. (24) report on a significant positive correlation between urinary 8-OHdG, a biomarker of oxidative DNA damage, and fasting blood glucose and HbA1c. In the present study, we found a significant positive correlation between DNA damage and HbA1c. Hyperglycemia causes glucose auto-oxidation, glycation of proteins, activation of polyol metabolism and subsequent formation of ROS. It has also been demonstrated that hyperglycemia is associated with an increased production of free radicals in the mitochondria and may contribute to a greater DNA damage (25).

We did not find correlation between duration of diabetes and DNA damage. The lack of correlation with duration of diabetes has been reported by other authors, in diabetes (23) and in chemical exposure (26,27), and investigators suggest that long-term

<table>
<thead>
<tr>
<th>Variable</th>
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<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.06</td>
<td>0.8</td>
</tr>
<tr>
<td>BMI</td>
<td>0.05</td>
<td>0.76</td>
</tr>
<tr>
<td>Duration</td>
<td>0.062</td>
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</tr>
<tr>
<td>Fasting blood glucose</td>
<td>0.81</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>0.5</td>
<td>0.003*</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.16</td>
<td>0.41</td>
</tr>
<tr>
<td>Triglycerides</td>
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<td>0.26</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>0.14</td>
<td>0.45</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>0.26</td>
<td>0.17</td>
</tr>
</tbody>
</table>

*p is significant; BMI = body mass index; HDL = high density lipoprotein; LDL = low density lipoprotein.
chronic exposure causes adaptation of response to damage and produces less genetic damage than initial exposure (26,27). On the other hand, another study found a positive association between duration of diabetes and expression of the enzyme DNA glycosylase 8-OxoG DNA glycosylase (Ogg1), which is a major repair enzyme involved in defense against accumulation of the 8-OHdG adducts (28).

One of the complications of diabetes is atherosclerosis. Atherosclerosis is associated with DNA damage that increases with progression of atherosclerosis. Recent studies revealed increased DNA damage in obesity with significant positive correlation with cholesterol, triglycerides and LDL-c, and DNA damage was present in atherosclerotic plaques and in circulating cells of patients with atherosclerosis (29,30). It is not known whether DNA damage in diabetes directly promotes atherosclerosis, or it is a byproduct of dyslipidemia of diabetes. Some cholesterol oxidation products (oxysterols) lead to the generation of reactive oxygen/nitrogen species (ROS/RNS) that can cause DNA damage; also, ROS are involved in oxidation of LDL, which is considered a fundamental step in the initiation and progression of atherosclerosis (31). In the current study, we did not find significant correlation between DNA damage and serum total cholesterol, HDL-c, LDL-c, triglycerides and BMI. However, a recent study revealed increased DNA damage as assessed by serum 8-OHdG level, in obese and overweight subjects compared to lean subjects (32). Another study revealed positive correlation between DNA damage, as assessed by comet assay, and total cholesterol, triglycerides and LDL-c in obese non-diabetic subjects (33).

In conclusion, type 2 diabetic patients have more oxidative DNA damage than normal controls and poor glycemic control may aggravate this damage. Dyslipidemia is not a contributing factor for DNA damage in diabetes.

REFERENCES


