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Oxidative Damage to DNA and Its Relationship With Diabetic Complications[1]

HONG-ZHI PAN[#, 2], DONG CHANG[+], LEI-GUANG FENG[+], FENG-JUAN XU[+], HONG-YU KUANG[+], AND MING-JUN LU[#]

[#]*School of Public Health, Harbin Medical University, Harbin 150081, Heilongjiang, China;*
[+]*The First Affiliated Hospital of the Harbin Medical University, Harbin 150001, Heilongjiang, China*

Objective To detect the oxidative DNA damage in diabetic patients and to investigate the relationship of oxidative DNA damage with diabetes and diabetic nephropathy. **Methods** Single cell gel electrophoresis (SCGE) was used to detect the DNA strand breaks in peripheral blood lymphocytes, and oxidative DNA damage product and serum 8-OHdG were determined by a competitive ELISA in 47 cases, including 25 patients without diabetic complications, 22 patients with diabetic nephropathy and 25 normal control subjects. **Results** Diabetic patients showed greater oxidative damage to DNA. The percentage of comet cells and the length of DNA migration (comet tail length) of peripheral blood lymphocytes were significantly increased in patients with diabetes, and significantly higher in patients with diabetic nephropathy than in diabetic patients without vascular complications ($P<0.05$). There was a significant increase in serum 8-OHdG in diabetic patients compared with normal subjects ($P<0.05$). Moreover, serum 8-OHdG was much higher in patients with diabetic nephropathy than in diabetic patients without vascular complications ($P<0.05$). **Conclusion** There is severe oxidative DNA damage in diabetic patients. Enhanced oxidative stress may be associated with diabetes, especially in patients with diabetic nephropathy.

Key words: Diabetes; Diabetic nephropathy; Comet assay; 8-OHdG

INTRODUCTION

Diabetes is a chronic metabolic syndrome due to insulin deficiency. Vascular complications are the leading cause of morbidity and mortality in diabetic patients. Diabetes-associated nephropathy is one of the major chronic complications of type 2 diabetes and finally progresses to end-stage renal disease requiring dialysis therapy.

The pathogenesis of diabetic nephropathy is multi-factorial, and the precise mechanisms are unclear. Several mechanisms have been proposed, such as increased production of advanced glycation end products (AGEs)[1], enhanced polyol pathway[2], activation of protein kinase C[3], and enhanced oxidative stress[4]. A number of studies *in vitro* and *in vivo* suggest that oxidative stress is increased in diabetic patients and animal models[5-7]. Oxidative stress may contribute to the pathogenesis of diabetic nephropathy. However, detailed molecular mechanism remains uncertain.

Single cell gel electrophoresis (SCGE) or comet assay, is a sensitive, simple, inexpensive, and rapid method. and can be used to detect DNA damage of the individual cells and reveal the presence of double-strand breaks, single-strand breaks and alkali-labile sites[8-9]. In recent years, this method has been widely used in studies on DNA repair, genetic toxicology, radiation, pollution, aging, and others[8-12].

In general, oxidative stress, including reactive oxygen species (ROS), can damage cellular macromolecules, leading to DNA and protein modification and lipid peroxidation. Production of ROS and lipid peroxidation are increased in diabetic patients[13]. Oxidative stress may play an important role in the development of complications in these patients. ROS can cause strand breaks in DNA and base modifications including oxidation of guanine residues to 8-hydroxydeoxyguanosine (8-OHdG), an oxidized nucleoside of DNA, which is the most frequently detected and studied DNA lesion. It has

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[2]Correspondence should be addressed to Hong-Zhi PAN, School of Public Health, Harbin Medical University, 157 Baojian Road, Nangang District, Harbin 150081, Heilongjiang, China. Tel: 86-451-87502725. Fax: 86-451-87502885. E-mail: panhongzhi@163.com
Biographical note of the first author: Hong-Zhi PAN, male, born in 1969, M. D., associate professor, majoring in nutrition and diabetes.

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been regarded as a novel biomarker of oxidative DNA damage *in vivo*. In humans, higher levels of 8-OHdG are observed in mononuclear cells from diabetic patients[14].

The aim of this study was to investigate the relationship of oxidative DNA damage with diabetes and diabetic nephropathy. We used the comet technique to calculate the frequency of comet in and comet tail length of peripheral blood lymphocytes, and measured the content of serum 8-OHdG by ELISA assay in patients with type 2 diabetes and in healthy control subjects.

MATERIALS AND METHODS

Subjects

Forty-seven patients with type 2 diabetes (23 men and 24 women, with median age of 58 years ranging from 42 to 72 years) were recruited. Twenty-five healthy, age-matched subjects (13 men and 12 women, with median age of 58 years ranging from 45 to 67 years, group C) served as the control. The diabetic patients included 25 patients without diabetic complications (group A, 12 men and 13 women, with median age of 58 years ranging from 42 to 70 years) and 22 patients with diabetic nephropathy (group B, 11 men and 11 women, with median age of 59 years ranging from 48 to 72 years). All the patients were diagnosed according to the World Health Organization diagnostic criteria for type 2 diabetes. We excluded the patients who had acute and chronic infections, fever, malignancy, acute and chronic nephritis, cirrhosis, and congestive heart failure. All the patients were under stable conditions when they were assessed.

Measurement of Comet Assay

Preparation of cell microgels on slides To detect DNA damage in individual cells, peripheral blood lymphocytes were analyzed by comet assay. All the procedures for comet assay were done at low temperature to minimize spontaneous DNA damage. Cell microgels were prepared as layers. The first layer of gel was made by applying 90 μL of regular melting point agarose (0.7%) onto glass microscope slides and a coverslip was put gently on the agarose. The coverslip was removed after the agarose solidified at 4℃. Low melting-point agarose was prepared in 100 mmol/L PBS and kept at 37℃. Samples of peripheral blood lymphocytes were mixed with the low melting-point agarose and 90 μL of the mixture was applied to the first gel layer. The slides were then covered with a coverslip and placed at 4℃ for solidification. After the second layer solidified, the coverslips were removed from the cell microgels.

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 lauryl sarcosine, 10 mmol/L Tris, 1% Triton X-100, 10% DMSO (pH 10)) at 4℃ for 1 h. After draining, microgels were treated with DNA unwinding solution (300 mmol/L NaOH, 1 mmol/L EDTA, pH 13) for 30 min at 4℃, and placed directly into a horizontal gel electrophoresis chamber filled with DNA-unwinding solution. Gels were run with constant current (100 mA at 4℃) for 30 min. After electrophoresis, the microgels were neutralized with 50 mmol/L Tris-HCl (pH 7.5) for 5 min. The slides were stained with 20 μL ethidium bromide (10 μg/mL), and viewed under fluorescence microscope (Olympus Vanox, Japan).

Analysis of Comet Slides

Each cell had the appearance of a comet, with a brightly fluorescent head and a tail to one side formed by the DNA containing strand breaks that were drawn away during electrophoresis. Percentage of comet cells and comet tail length were calculated with Komet image analysis system (Kinetic Imaging Ltd., UK). Samples were run in duplicate, and 50 cells were randomly analyzed per slide for a total of 100 cells per sample.

Measurement of Serum 8-OHdG

Serum 8-OHdG was determined by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (Highly sensitive 8-OHdG check, Institute for the Control of Aging, Fukuroi, Shizuoka, Japan). The sera were diluted with PBS (pH 7.4) and filtered using ultra filter following the manufacturer's instructions before the measurement. The lower limit of detection for 8-OHdG was 0.125 ng/mL. Monoclonal antibody, N45.1, with an established specificity, was used as a primary antibody. Optical density was measured at 450 nm using a microplate reader (Triturus, Spain). Quantification of 8-OHdG was achieved by comparing the optical densities of each sample to that of an internal standard of known 8-OHdG at various concentrations.

Statistical Analysis

Data are presented as $\bar{x} \pm s$. All experimental data in this study were statistically analyzed with SPSS13.0. Hypothesis testing methods included unpaired Student's *t*-test and chi square test (χ^2 test).

$P<0.05$ was considered statistically significant.

RESULTS

Results of Comet Assay

SCGE assay revealed that the number of DNA strand breaks was significantly higher in cells of the diabetic patients than in cells of the control. Most cells (86%, Fig. 1A) in diabetic nephropathy patients contained DNA strand breaks, which was higher than in diabetic patients without vascular complications (29%, $P<0.01$, Fig. 1B). In the control group, lymphocytes with DNA strand breaks were scarce or completely absent (Fig. 1C). The comet tail length was significantly increased in group B compared with group A (15.12±4.39 μm *vs* 12.49±3.40 μm, $P<0.05$).

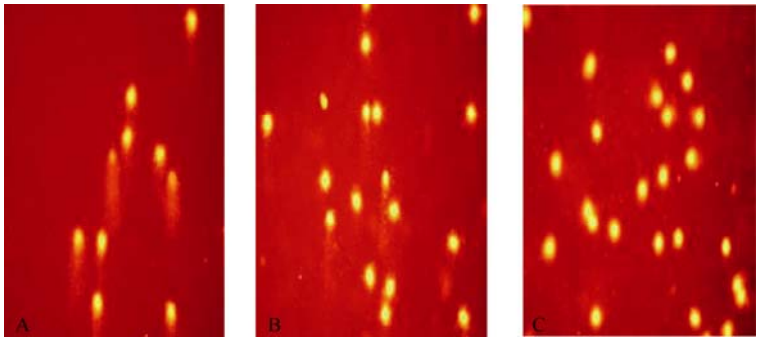

FIG. 1. Comet image of peripheral blood lymphocytes showing DNA strand breaks in patients with diabetic nephropathy (A), in diabetic patients without vascular complications (B), and in healthy control (C).

8-OHdG in Serum

Serum 8-OHdG was significantly increased in diabetic patients (24.90±6.29 ng/mL) compared with age-matched normal subjects (5.86±1.82 ng/mL, $P<0.01$). Moreover, patients with diabetic nephropathy also had a significantly higher 8-OHdG than diabetic patients without vascular complications (26.89±6.44 ng/mL *vs* 23.14±5.59 ng/mL, $P<0.05$).

DISCUSSION

Diabetes mellitus may be associated with increased lipid peroxidation caused by oxidative stress. Increasing evidence from both experimental and clinical studies suggests that there is a close link between hyperglycemia, oxidative stress and diabetic complications. It has been reported that oxidative stress contributes to the pathological processes of diabetic complications, including nephropathy[15]. However, its mechanism remains to be elucidated. This study showed that diabetic patients, especially patients with nephropathy had a certain degree of oxidative DNA damage.

SCGE is a commonly used method to detect oxidative damage in lymphocyte DNA and the degree of oxidative stress is related to the size of comet tail[16]. This highly sensitive assay is able to detect very low levels of damage. In the present study, the number of DNA strand breaks was significantly increased in diabetic patients with nephropathy compared with those without vascular complications and healthy control. High blood glucose levels *in vitro* impair cellular DNA repair and increase DNA cleavage[17]. Hyperglycemia itself contributes to increased generation of ROS and increased oxidative stress would lead to oxidative DNA damage. Nishikawa *et al.*[17] showed that blockade of hyperglycemia-induced ROS production would reverse the pathways implicated in diabetic angiopathy in cultured endothelial cells.

Increased oxidative stress plays an important role in the progression of diabetic nephropathy. In general, oxidative stress can affect nucleic acids and generate various modified bases in DNA. 8-OHdG is one of the most abundant oxidative products of DNA and appears to play a crucial role in mutagenesis. It is released into blood and urine after excised from DNA by the repair enzyme. Previous studies showed that diabetic patients have higher levels of 8-OHdG in mononuclear cells, urine, pancreatic islet and mitochondrial DNA[14,18-23]. Kakimoto *et al.*[24] showed that the levels of 8-OHdG are increased in kidney tissues of streptozotocin-induced diabetic rats[24]. Moreover, the contents of 8-OHdG in urine and mononuclear cells of type 2 diabetic patients with either retinopathy or nephropathy are much higher than those in patients without complications[6]. Urinary 8-OHdG excretion, a sensitive biomarker of oxidative DNA damage, is significantly correlated with severity of tubulointerstitial lesions in diabetic nephropathy[25]. In the present study, we observed a similar association of serum 8-OHdG with diabetic

nephropathy, suggesting that serum 8-OHdG can also act as a sensitive biomarker for diabetic nephropathy.

In conclusion, diabetic patients have more severe oxidative DNA damage than normal persons. Levels of oxidative DNA damage are increased in diabetic patients, suggesting that increased oxidative stress may be associated with the progression of diabetes, especially in diabetic patients with nephropathy. Assessment of oxidative stress in diabetic patients may be important in predicting and preventing oxidative stress-related complications.

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Correction

There is a mistake in page 24 of Volume 20, No.1. The address "[#]Department of Life Sciences, National Taiwan Normal Universities, Sec. 4 Tingchou Rd, Taipei 116, Taiwan" written in page 24 should be corrected by "[#]Department of Life Sciences, National Taiwan Normal Universities, Sec. 4 Tingchou Rd, Taipei 116, Taiwan, China".

Hereby statement!