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Ginkgo biloba attenuates oxidative DNA damage of human umbilical vein endothelial cells induced by intermittent high glucose

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Received August 24, 2013, accepted September 23, 2013

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Pharmazie 69: 203–207 (2014) *doi: 10.1691/ph.2014.3819*

Intermittent high glucose (IHG), one of the general and important symptoms of patients with diabetes, has greater effect than sustained high glucose on the development of diabetic cardiovascular complications, in which endothelial dysfunction caused by oxidative stress is regarded as the initiation. However, no study investigated either the degree of endothelial DNA oxidation caused by IHG or the potential protective effects of antioxidants. In this study, DNA oxidation, including 8-hydroxy-2'-deoxyguanosine (8-OHdG) concentration and comet assay, was studied in human umbilical vein endothelial cells (HUVECs) under IHG with or without treatment of *Ginkgo biloba* extract (EGb 761). We found that high glucose, especially IHG, increased reactive oxygen species generation, 8-OHdG content and oxidative DNA damage in HUVECs. These high glucose-induced oxidative stress could be suppressed by EGb 761 (25–100 μg/ml) in a dose-dependent manner through the improvement of total antioxidant capacity. Our results indicated that the presence of significant DNA oxidation in HUVECs exposed to high glucose, and especially higher in the cells in IHG conditions. EGb 761, an antioxidant herbal medicine, can remarkably alleviate endothelial DNA oxidation caused by IHG, which may provide a novel approach for endothelial protection in the presence of IHG.

1. Introduction

Diabetes mellitus can cause a wide variety of vascular complications and it is one of the major risk factors of atherosclerosis (Zhang et al. 2012). The vascular endothelium is a single cell layer lining the vascular wall and plays an important role in maintaining the structure and function of vessels. The formation of atherosclerotic lesions is caused by sublethal changes in endothelial function, called endothelial dysfunction, in presence of various stimulators (Vita 2011). Among them, oxidative stress is well documented to act as a serious causative factor and plays a critical role in the pathogenesis of several vascular diseases (Lum and Roebuck 2001). Oxidative stress, defined as the loss of balance between reactive oxygen species (ROS) production and antioxidant defenses, is considered to cause oxidative damage to lipids, proteins and DNA. It is now recognized that the interaction of ROS with DNA results in an impairment of the genetic material in the cell nucleus. In the absence of an adequate antioxidant system, accumulated DNA damages may result in dysfunction of relevant cells. It is acknowledged that oxidative DNA damage is a cause of both initiation and progression of atherosclerosis, and not just a consequence of disease (Gray and Bennett 2011). Many experiments have shown that the major stimulus inducing DNA damage in atherosclerosis is oxidative stress due to ROS, the specific risk factor for cardiovascular diseases (Gray and Bennett 2011).

Besides chronic persistent hyperglycemia, repeated fluctuation from euglycemia to hyperglycemia, or intermittent high glucose (IHG), is another general and important symptom in patients with diabetes (Cho et al. 2006). Recently, several studies demonstrated that IHG has greater effect than sustained high glucose (SHG) on the development of diabetic cardiovascular complications, via enhancement of monocyte adhesion to endothelial cells, generation of ROS and other mechanisms (Quagliaro et al. 2006; Piconi et al. 2004; Ge et al. 2010). However, little is known about the toxic effect of IHG on DNA oxidation of endothelial cells.

Ginkgo biloba leaves, one of the oldest herbal medicines, have been used as a therapeutic agent for some cardiovascular and neurological disorders in modern pharmacology (Rodríguez et al. 2007; Luo 2006). *Ginkgo biloba* extract (EGb 761), a standardized extract of *Ginkgo biloba* leaves, was shown a variety of biological activities. Among them, the antioxidant properties of EGb 761 have been intensively examined as a potential mechanism for its beneficial effect (Eckert et al. 2003). EGb 761 protects the heart against ischemia reperfusion

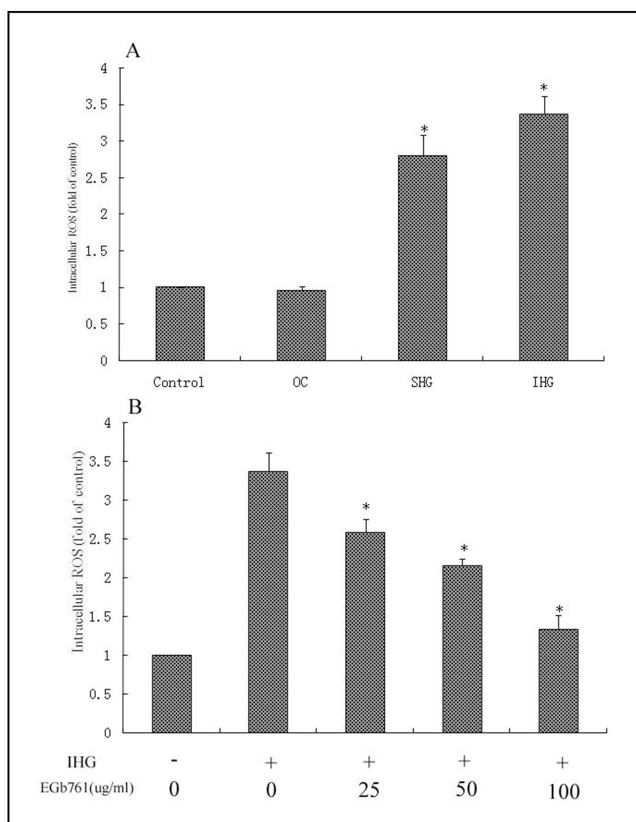

Fig. 1: Effect of EGb 761 on the level of intracellular ROS induced by IHG in cultured HUVECs. After 72 h incubation, intracellular ROS production was detected by the fluorescence of 2', 7'-dichlorofluorescin diacetate. (A) High glucose, especially IHG, increased more ROS generation in HUVECs. (B) EGb 761 (25–100 μg/ml) dose-dependently decreased ROS generation in HUVECs exposure to IHG. Data were presented as mean ± SD from 3 independent experiments and every experiment was repeated five times. *$P < 0.01$ vs. control.

injury and endothelial dysfunction evoked by ROS (Akiba et al. 2004; Fan et al. 2006). *Ginkgo* reduced generation of ROS and inflammatory protein expression in oxidized low-density lipoprotein-stimulated human vascular endothelial cells (Zhang et al. 2011). Furthermore, EGb 761 can protect against intermittent hypoxia induced memory impairment, oxidative stress and neuronal DNA damage (Abdel-Wahab and Abd El-Aziz 2012). Lim et al. (2011) reported that EGb 761 is effective against atherosclerosis both *in vitro* and in a rat model of type 2 diabetes. However, it is not clear whether it can directly inhibit DNA oxidation of endothelial cells caused by ROS such as induced by IHG.
Therefore, to investigate the mechanism of IHG on DNA oxidation of endothelial cells and the potential protective effect of *Ginkgo biloba*, we detected oxidative DNA damage in human umbilical vein endothelial cells (HUVECs) exposed to IHG, and assessed whether EGb 761 could attenuate these damages.

2. Investigations and results

2.1. EGb 761 inhibited IHG-induced ROS generation in HUVECs

Intracellular ROS production increased in sustained high glucose-treated cells by about 250% compared to cells under normal glucose exposure, and further increased under intermittent glucose conditions ($P < 0.01$) (Fig. 1A). EGb 761 (25–100 μg/ml) treatment significantly decreased the level of ROS in a dose-dependent manner for HUVECs exposure to IHG (all $P < 0.01$) (Fig. 1B).

2.2. EGb 761 ameliorated DNA oxidation in HUVECs

To further determine the effect of IHG on DNA oxidation of HUVECs and protective effect of EGb 761, 8-hydroxy-2'-deoxyguanosine (8-OHdG) content and comet assay (Single-cell micro gel electrophoresis) were performed. As shown in Fig. 2A , the level of 8-OHdG increased in cells exposed to stable high glucose and substantially more in cells exposed to the intermittent high glucose concentrations ($P < 0.01$). EGb 761 dose-dependently reduced 8-OHdG production in intermittent glucose states (Fig. 2B). Similar changes were detected in the comet assay. DNA damage in HUVECs (comet %) was 2.4-fold and 3.0-fold higher in SHG and IHG than in control respectively (Fig. 2C), and decreased with increasing concentrations (25–100 μg/ml) of EGb 761 (Fig. 2D) ($P < 0.01$).

2.3. EGb 761 improved the intracellular total antioxidant capacity (TAC) content

In the absence of EGb 761 in the medium, TAC contents were decreased in cells cultured with constant high glucose, and to a greater extent under conditions of IHG ($P < 0.01$). EGb 761 dose-dependently improved the level of TAC in IHG conditions ($P < 0.01$) (Fig. 3). Furthermore, intracellular TAC was negatively correlated to both 8-OHdG content ($r = -0.776$, $P = 0.003$, Fig. 4A) and comet % ($r = -0.741$, $P = 0.006$, Fig. 4B).

3. Discussion

In this study, we showed that a 72-h exposure to IHG induced more serious DNA oxidation and reduced total antioxidant capacity than SHG in cultured HUVECs *in vitro*. In addition, reduced TAC was negatively correlated to the increased DNA oxidation which detected by both 8-OHdG content and comet assay. However, treatment with EGb 761 (25–100 μg/ml) suppressed the elevation of DNA oxidation induced by high glucose in a dose-dependent manner.
Reactive oxygen species, which derived from the metabolism of oxygen, are key modulators in regulating different steps in vascular development (Zhou et al. 2013). However, an imbalance between oxidant and antioxidant status, resulting either from increased production of ROS or inactivation and excessive consumption of antioxidant systems, causes oxidative stress. Under conditions of oxidative stress, cellular biomolecules such as lipids, proteins, and DNA become damaged and participate in many pathologic processes (Floyd et al. 2011). For example, increased ROS formations and accumulation of DNA damage are widely recognized to participate in both initiation and progression of atherosclerosis characterized by the generation of endothelial dysfunction (Gray and Bennett 2011).
Generally, endothelial dysfunction in diabetes is characterized by impairment of endothelial cell functions and survival induced by chronic sustained high glucose. Although plasma glucose concentration is strictly controlled within a narrow range in normal subjects, the plasma glucose concentration in patients with pre-diabetes or diabetes often changes markedly during the period of a single day (Cho et al. 2006), which is usually accompanied by activation of oxidative stress (Monnier et al. 2006). Several previous studies have shown that IHG induced endothelial cells apoptosis and up-regulated adhesion molecules and inflammation related to oxidative stress (Quagliaro et al. 2005; Ge et al. 2010; Quagliaro et al. 2003; Piconi et al. 2006). However, there is little evidence focused on the toxic effects of the periodical hyperglycemic spikes on oxidative DNA damages of endothelial cells.
Comet assay—a rapid, simple, and sensitive technique for measuring and analyzing DNA breakage in single mammalian

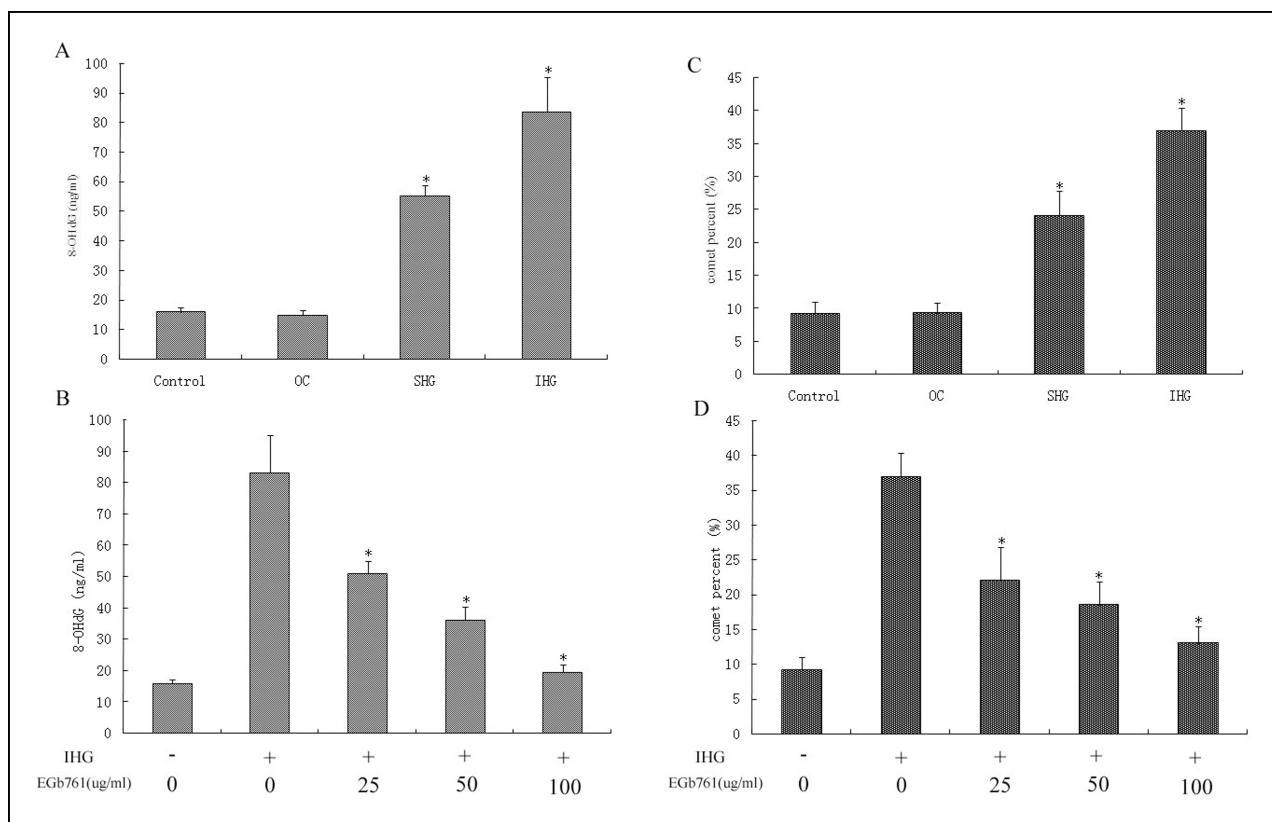

Fig. 2: Inhibitory effects of EGb 761 on IHG-induced DNA oxidation in HUVECs. After 72 h incubation, 8-OHdG content and comet assay were performed as described in material and method. High glucose, especially IHG, increased 8-OHdG (A) and comet percent (C) in HUVECs, while EGb 761 (25–100 μg/ml) dose-dependently ameliorated above changes (B and D). Data are presented as mean ± SD from 3 independent experiments and every experiment was repeated five times. *$P < 0.01$ vs. control.

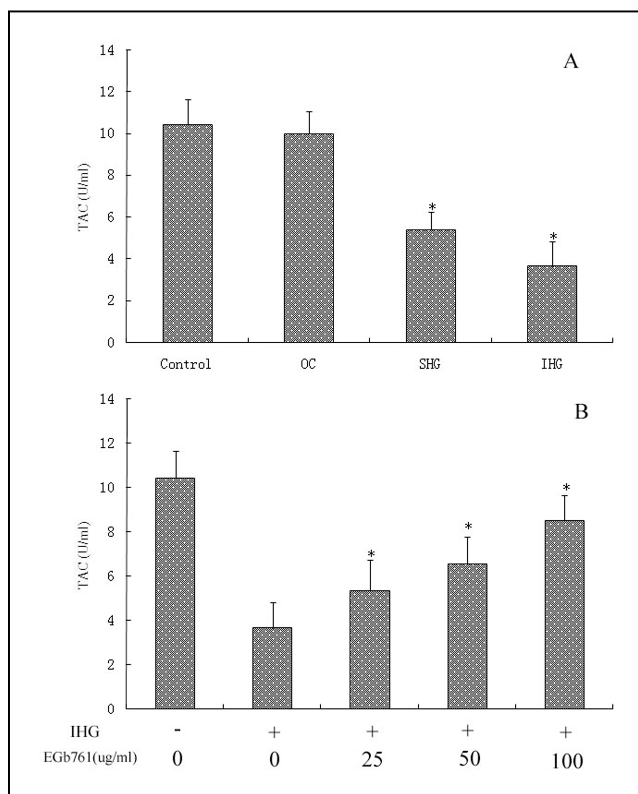

Fig. 3: Effect of EGb 761 on intracellular TAC of HUVECs explored to IHG. After 72 h incubation, intracellular TAC content was detected. (A) High glucose, especially IHG, reduced intracellular TAC of HUVECs. (B) EGb 761 (25–100 μg/ml) dose-dependently improved intracellular TAC in HUVECs exposure to IHG. Data are presented as mean ± SD from 3 independent experiments and every experiment was repeated five times. *$P < 0.01$ vs. control.

cells—is widely used to investigate the effects of ROS on DNA. In this study, we selected the comet assay and determined the concentrations of 8-OHdG, another sensitive and specific marker of oxidative DNA damage, to evaluate the levels of DNA oxidation induced by IHG. Our results showed that both markers were significantly increased in HUVECs incubated with SHG, which was more pronounced with IHG exposure. Meanwhile, our results also showed a decline in TAC in high glucose incubated HUVECs, especially those cultured in IHG. As an index of the capacity of a given tissue to modulate the damage associated with increased production of free radicals, TAC is widely used to assess the degree of oxidative stress under many pathologic conditions (Somogyi et al. 2007). Thus, our results provide evidence that more serious DNA oxidation of endothelial cells induced by IHG may be due to the less capacity of antioxidant. *Ginkgo biloba*, a Chinese herb, has been used in traditional Chinese medicine for thousands of years. *Ginkgo biloba* extract (EGb) is extracted from *Ginkgo biloba* leaves and is a well-defined complex mixture containing 24% Ginkgo flavone glycoside and 6% terpen lactones (ginkgolides, bilobalide). The standard preparation EGb 761 has been used as a therapeutic agent for some cardiovascular and neurological disorders through its purported biological effects such as scavenging free radicals, lowering oxidative stress, reducing neural damages, reducing platelet aggregation, anti-inflammation, anti-tumor activities, and anti-aging (Chan et al. 2007). More recently, Chen et al. (2012) showed that EGb 761 could reduce high-glucose-induced endothelial ROS generation and cell adhesion molecule expression through its antioxidant capacity. In this study, our results further confirmed that EGb 761 could reduce not only SHG but also IHG induced DNA oxidation of endothelial cells. Our data confirmed that the potential mechanisms involved in the toxic effects of IHG on DNA oxidation of endothelial cells are, at

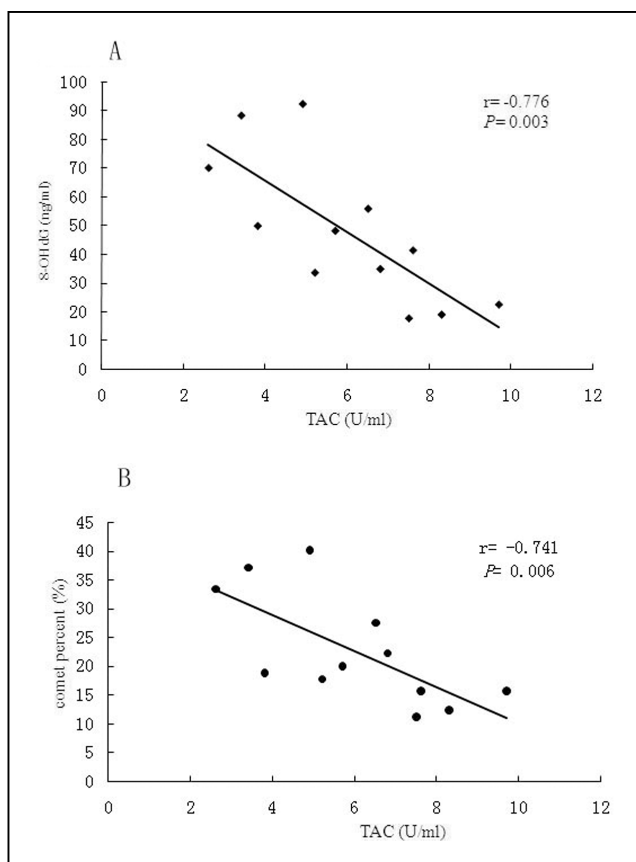

Fig. 4: Relationship between intracellular TAC and DNA oxidation of HUVECs. DNA oxidation of HUVECs were detected by 8-OHdG content and comet assay. Negative correlations were shown between intracellular TAC and 8-OHdG content (A) and comet assay (B) of HUVECs in IHG conditions treated with different doses of EGb 761.

least in part, the same as those working in SHG concentrations. However, they are consistently enhanced in such a condition. Until now, the molecular mechanisms specially triggered on cultured endothelial cells by periodically variable high glucose concentrations are not known. A possible explanation is that during chronic exposure to high glucose, some metabolic changes induced by this sustained situation might change feedback regulation of the cells, partially counteracting glucose toxicity (Hou et al. 2008). Intermittent exposure to high glucose might reduce such adaptation, causing more pronounced toxic effects (Hou et al. 2008). This speculation needs to be elucidated by further investigations.

In summary, our study suggested that exposure to IHG led to more serious DNA damage and reduced total antioxidant capacity of endothelial cells than SHG. EGb 761 treatment reduced IHG-induced DNA oxidation of endothelial cells. Given that vascular complications are a major cause of morbidity and mortality in diabetes, our study provide a novel therapeutic approach for endothelial protection in the presence of IHG. Certainly, further studies are needed to clarify the cellular and molecular mechanisms of EGb 761.

4. Experimental

4.1. Cell culture and treatment

HUVECs were obtained from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. The HUVECs cells from passage 10 to 20 were seeded at equal density and cultured in RPMI 1640 medium containing 11.1 mmol/l glucose, supplemented with 10% fetal calf serum, pH 7.4 at 37 °C and 5% CO_2 according to the manufacturer's instructions. In experiments, HUVECs were rinsed twice with PBS, and then randomly divided into different groups, and exposed to 11.1 mmol/l glucose (control), or 25.0 mmol/l glucose (SHG), or 11.1 and 25.0 mmol/l glucose alternating every 12 h (IHG) (Hou et al. 2008), and maintained for 72 h, respectively. An equal amount of mannitol was used as an osmotic control (OC). All media were changed every day. To determine the protective effect of *Ginkgo biloba*, HUVECs were treated with RPMI 1640 medium containing EGb 761 (25, 50 or 100 μg/ml) (Pierre et al. 2008), a standardized commercially-available preparation of *Ginkgo* extract (Beaufour IPSEN Institute, Paris, France) for 72 h in the presence of IHG.

4.2. Detection of intracellular ROS

The level of intracellular ROS was detected using 2',7'- dichlorofluorescein diacetate (DCFH-DA) as a probe (Beyotime, Jiangsu, China). Briefly, cells (3×10^5 cells/ml) were treated for 72 h, then washed twice and incubated with final concentration of 10 μM DCFH-DA for 30 min at 37 °C in the dark. After incubation, cells were washed once and harvested in PBS. The fluorescence of 2',7'-dichlorofluorescein (DCF) was detected with a flow cytometer (Coulter Epics XL, Beckman-Coulter, USA) at an emission wave length of 525 nm and excitation wave length of 488 nm. Data were processed by using cell quest software (BD Biosciences, USA).

4.3. Measurement of TAC

TAC in supernatants of treated HUVECs were 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 (Benzie and Strain 1996). One unit of TAC is represented by an increase in the absorbance of the reaction mixture of 0.01 per ml of sample per min. The measurement was performed with a commercial test kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. At least three independent experiments were performed in all samples, and every experiment was repeated five times. Results are expressed as units/ml of supernatant.

4.4. Determination of 8-OHdG concentration

8-OHdG content in HUVECs (4×10^6 cells/ml in 100 × 100 mm dishes) was measured using the Bioxytech 8-OHdG-EIA Kit, a competitive enzyme-linked immunosorbent assay (ELISA) purchased from OXIS Health Products (Portland, OR, USA). HUVECs DNA was isolated using DNA-zol reagent according to the manufacturer's instructions, and quantified using a spectrophotometer at OD260/OD280. Samples containing 200 μg DNA were resuspended in 50 μl of reaction mixture containing 100 mmol/l sodium acetate (pH 5.0) and 5 mmol/l $MgCl_2$, and then they were digested with 1 μl DNase I for 10 min at room temperature. DNA-digested samples were added to the microtiter plate pre-coated with 8-OHdG, and the assay was performed according to the manufacturer's instructions. OD450 was determined with a microplate reader.

4.5. Single-cell micro gel electrophoresis

To determine the DNA damage in cultured HUVECs, single-cell micro gel electrophoresis assay, or comet assay as a synonym, was performed under alkaline conditions as previously reported (Wang et al. 2012). Treated HUVECs were suspended in agarose and spread into a glass microscope slide pre-coated with agarose. Agarose was allowed to set at 4 °C for 5 min. Slides were incubated in ice-cold lysis solution (2.5 MNaCl, 100 mM EDTA, 10 mM Tris, pH 10.0, and 1% triton X-100 with 10% DMSO) to remove cell proteins, leaving DNA as 'nucleoids'. After the lysis procedure, slides were placed on a horizontal electrophoresis unit, covered with a fresh solution (300 mM NaOH and 1 mM EDTA, pH > 13) for 20 min at 48 °C to allow DNA unwinding and the expression of alkali-labile sites. Electrophoresis was conducted in an electrophoretic solution containing 30 mM NaOH and 1 mM EDTA, pH > 13 at an ambient temperature of 4 °C (the temperature of the running buffer did not exceed 12 °C) for 20 min at an electric field strength of 0.73 V/cm (28 mA). The slides were then washed in water, drained, and stained with ethidium bromide, and covered with cover slips. To prevent additional DNA damage, all the steps were conducted under dimmed light or in the dark. The comets were viewed at 200 × magnification with a fluorescent microscope [BH-2 Olympus, excited by green light (546 nm) and barrier filter of 590 nm] within 24 h, and photomicrographs taken. For each sample, three slides were scored for, with at least 100 cells counted randomly on each slide. No DNA-damaged cells retained the circular appearance. DNA with strand breaks migrated towards the anode during electrophoresis; and the cell appeared as a comet. According to Everett et al. (2000), the slides were analyzed under blind conditions at least by two different individual, median values of the scores were presented. We used readings mean of the scores which was agreement.

4.6. Statistical analysis

Statistical analysis was performed with Statistical Package for the Social Science (version 12.0; SPSS; Chicago, USA) SPSS 12.0. Quantitative variables are presented as mean ± SD. Paired t test was used to analyze continuous normally distributed variables. Correlation analysis was performed with linear correlation. A 2-tailed $P < 0$ .05 was considered statistically significant.

Acknowledgements: We thank Dr. Nan Wu (St. Boniface Hospital Research Centre; Department of Physiology, University of Manitoba, Canada) for professional English editing. This research was funded by the Young Scientists Star Program of Jinan City (grant no. 09114), Natural Science Foundation (grant No. ZR2009CQ023) and Medical Science Development Plan (grant No. 2009QZ025) of Shandong Province, PR China.
Disclosure: The authors declare that they have no competing interests.

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