Assessment of DNA Strand Breakage in Streptozotocin-induced Diabetic Rats

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ABSTRAK

Kajian ini telah dijalankan untuk mengkaji kerosakan oksidatif pada tikus aruhan diabetes melitus. Evaluasi aras kerosakan DNA dilakukan dengan kaedah Asai Komet Alkali menggunakan sel limfosit periferi yang diambil daripada darah tikus teraruh diabetes oleh streptozotocin (50 mg/kg) dan tikus kawalan. Aras malondialdehid (MDA), 4-hidroksinonenal (4-HNE), glukos darah puasa (FBG) dan HbA1c turut diukur. Semua tikus yang diaruh diabetes disahkan mengalami hiperglisemia sehingga akhir kajian dengan aras FBG dan HbA1c adalah lebih tinggi secara signifikan berbanding tikus kawalan. Hasil ujian juga mendapati peratusan ekor DNA dan nilai 'tail moment' juga tinggi secara signifikan bagi tikus teraruh diabetes. Pemerhatian yang sama turut dilakukan untuk aras MDA dan 4-HNE. Sebagai kesimpulan, hasil kajian ini menunjukkan bahawa keadaan hiperglisemia pada tikus teraruh diabetes boleh menjana kerosakan oksidatif DNA.

Kata kunci: diabetes mellitus, hiperglisemia, kerosakan oksidatif, kerosakan DNA, Asai Komet Beralkali

ABSTRACT

This study was conducted to evaluate the oxidative damage in diabetic mellitus induced rats. The evaluation of DNA damage was carried out by the Alkaline Comet Assay using peripheral lymphocyte cells taken from streptozotocin-induced diabetic rats (50 mg/kg) and control rats. The levels of malondealdehyde (MDA), 4-hydroxynonenal (4-HNE), fasting blood glucose (FBG) and HbA1c were also measured. All the induced diabetic rats were hyperglycemic until the end of the study with significantly higher levels of FBG and HbA1c as compared to the control rats. The results showed the percentage of tail DNA and tail moment values were also significantly higher in the diabetic induced rats. The same observations were made on the levels of plasma MDA and 4-HNE. In conclusion, this study indicated that hyperglycemic condition in diabetic induced rats could generate oxidative DNA damage.

Key words: diabetes mellitus, hyperglycemia, oxidative stress, DNA damage, Alkaline Comet Assay

INTRODUCTION

Diabetes mellitus is a disorder characterized by hyperglycemia due to an absolute or relative deficiency of insulin and/or insulin resistance. It is one of the most common metabolic disease, affecting about 2.5-3% of the world population, and 7% or more in some countries (Froguel & Velho 2001). Diabetic patients are prone to long-term complications, such as retinopathy, cataract, atherosclerosis, neuropathy, nephropathy and impaired wound healing. Hyperglycemia has an important role in the pathogenesis of long-term complications and those with poor glucose control are very much at risk (UKPDS 1998).

A considerable amount of evidence suggested that oxidative stress may play an important role in the pathogenesis and complications of diabetes and this hypothesis has been largely proven for insulin dependent diabetes mellitus (IDDM) (Seghrouchni et al. 2002). Different mechanisms can be contributed to the enhanced oxidative stress in diabetic patients, particularly in poor glycemic control and hypertriglyceridamia (Martin-Gallan et al. 2003). Furthermore the mechanism that can contribute to the formation of free radicals in diabetic patients may include not only with the increase of non-enzymatic and auto-oxidative glycosylation, but also the metabolic stress resulted from the changes in energy metabolism, levels of inflammatory mediators and in the status of the antioxidant defense systems (Martin-Gallan et al. 2003).

Increase in oxidative stress can be indicated by elevated concentration of lipid peroxidation products such as tiobarbituric reactive substances in plasma (Lyons 1991). Transient changes in activities of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase are also observed in conjunction with increase plasma glucose levels in cases where control of diabetes is poor, perhaps reflecting direct inhibition of the enzymes by hyperglycemia (Collins et al. 1998). Elevated oxidative stress is considered to be a likely cause for atherosclerosis, the most significant complication in diabetes, and the most common cause of premature death (Dandona et al. 1996). Quantitative determination of the levels of oxidative stress is a valuable indicator for the degree of disease severity and effectiveness of treatment.

Oxidative stress affects biomolecules other than lipids i.e. protein and DNA. Evidence in the increase of oxidative DNA damage in diabetes patients has been reported (Dandona et al. 1996; Collins et al. 1998). The most common reported product of oxidative DNA damage in diabetes is 8-hydroxy-2′-deoxyguanosine (8OHdG) (Dandona et al. 1996). It can be generated by several different reactive oxygen species (ROS), including hydroxyl radicals, singlet oxygen, peroxyl radicals and peroxynitrite, which are able to produce DNA lesion as well as strand breakage. The single cell electrophoresis assay (SCGE assay) or also known as the Alkaline Comet Assay is a sensitive method for measuring the extension of DNA damage in individual cell (Singh et al. 1988). In the present study, the streptozotocin (STZ)-induced diabetic rats were used to determine the presence of oxidative DNA damage in diabetes mellitus.

MATERIALS AND METHODS

ANIMALS

Male Sprague Dawley rats weighing between 260-290 g, were supplied by the Animal House of Universiti Kebangsaan Malaysia, located at Institute for Medical Research (IMR), Kuala Lumpur. Animals were housed in plastic cages with floors covered with wood shavings, and two rats were housed in each cage. All animals were maintained on a balance diet and water *ad libitum* continuously. The study was approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC) with license no; Biomed/2001/Siti/17-May/047 and the UKMAEC guidelines were also followed. Rats were randomly assigned to two experimental groups. Diabetes was induced following overnight fasting by single intravenous injection of streptozotocin (STZ) (50 mg/kg) (Sigma, St Louis, MO, USA), which was freshly dissolved in normal saline (n = 8). The control group only received saline injections (NDM) (n = 7). At day three of induction, glucose concentration in tail vein blood was determined by using strip-operated blood glucose sensor (Companion 2, Medisense Ltd., Birmingham, UK). All the STZ injected rats possessed blood glucose levels >15.0 mmol/l. Food and water intake and body weight were also recorded once a week.

BIOCHEMICAL ANALYSIS

Following eight weeks of treatment, the rats were fasted overnight and blood was collected by cardiac puncture under deep anesthesia with diethyl ether. Blood was collected into tubes containing EDTA and kept on ice and immediately centrifuged at 3000 rpm for 20 min at 4°C. The plasma collected was stored at - 40°C until analysis. The plasma glucose levels were analyzed on the same day using enzymatic glucose-oxidase kits (Catalogue No. TR 15104, Trace Scientific, Melbourne, Australia.). The blood HbA1c was determined using method by Eross et al. (1984) and expressed as percentage of total hemoglobin. Plasma protein concentration was measured by the method of Bradford (1976). Plasma MDA and 4-HNE were assayed using the kit by Calbiochem (Catalogue No. 437634, Calbiochem's Lipid Peroxidation Assay, USA).

THE ALKALINE COMET ASSAY

Lymphocyte preparation

A total of fresh 2 ml whole blood was slowly transferred to an eppendorf tube containing the same volume of histopaque and centrifuged at 3000 rpm for 30 minutes at 4 °C. The resulting cell pellets were washed 3 times with Mg^{2+}/ Ca^{2+}-free phosphate-buffered saline (PBS) then re-suspended in 20 µl of PBS and stored at 4 °C in the dark (to minimize additional DNA damage), to be used on the same day for the Alkaline Comet Assay.

Slide preparation

The Alkaline Comet Assay procedure was carried out following the method of Singh et al. (1988). Essentially, 100 µl of 0.5% normal melting point agarose (Sigma, USA) was

pipetted onto frosted microscopic slides and allowed to solidify under the coverslip, which was then carefully removed. Next, 10 µl of lymphocyte suspension were suspended in 80 µl low melting point agarose gel; the cell suspension was rapidly pipetted onto the first agarose layer, and gently spread by placing a coverslip on top. This was allowed to solidify on an ice tray for 5 min. After removal of the coverslip, the slide was immersed in a freshly prepared lysing solution (2.5 mM NaCl, 100 mM EDTA, and 10 mM Tris, with 1% Triton-100) and incubated for 1 hour at 4°C. The slides were removed from the lysing solution, drained, and placed in a horizontal gel electrophoresis tank. The tank was filled with fresh, cold electrophoresis solution (1 mM EDTA and 300 mM NaOH) to a level approximately 0.25 cm above the slides. The slides were left in the solution for 20 min to allow the unwinding of the DNA and expression of alkali-labile damage before electrophoresis. Electrophoresis was conducted at 4°C for 30 min using 25 V and current of 300 mA. Following electrophoresis, the slides were washed (3X) in Tris buffer (0.4 M Tris, pH 7.5) to neutralize the excess alkali. Slides were later stained with 50 µl ethidium bromide (20 ug/ml;Sigma, USA).

Image analysis of slides

Slides were kept in a lightproof box containing PBS moistened tissues and viewed following overnight storing at 4°C. Observation were made using a Leitz Laborlux fluorescence microscope (Nikon) equipped with an epifluorescence mercury lamp source (excitation filter 515, barier filter 590nm) and X40 fluorescence objective (numerical aperture 0.85) and data were analyzed using a specialized single-cell gel (SCG) image analysis programmed (TriTex Comet Score™ (free comet score))

Analysis of DNA damage

The image analysis software provides a full range of densitometric and geometric parameters describing the complete Comet, as well as the head and Tail DNA portions. Since the Comet Assay reflects the displacement of fluorescence from the head to the tail in damage cells, the used of % Tail DNA, i.e. the percentage of total nuclear DNA that has migrated to the tail, and tail moment (% Tail DNA x length) as the parameter to quantify basal levels of DNA damage. Each slide was analyzed in duplicate and 50 cells per slide were scored.

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS statistical package version 11.0. All results were expressed as mean ± standard error of the mean (SEM). Normality of distribution was verified by Shapiro-Wilk test. The parameters were analyzed with independent t-test to compare the difference between groups. The difference between groups was considered significant for $p < 0.05$.

RESULTS AND DISCUSSION

All diabetic rats showed persistent hyperglycemia until the end of the study. Levels of FBG and HbA1c % were significantly higher ($p<0.05$) as compared to the control group (NDM). MDA and 4-HNE levels, the products of lipid peroxidation process, were also significantly higher ($p<0.05$) in diabetic rats (Table 1). Figure 1 shows the images of lymphocyte DNA in NDM and diabetic rats following the Comet Assay. The basal endogenous DNA damage measured by the mean tail DNA (%) of the lymphocytes in diabetic rats was significantly higher than in NDM rats ($p<0.05$) (Fig. 2). The tail moment, which is defined as the percentage of DNA at the tail multiplied by the length of migration, was also significantly higher in diabetic group as compared to the NDM ($p<0.05$) (Fig. 3).

Oxidative stress has been well documented as a characteristic feature of diabetes and has also been implicated in the development of complications associated with diabetes, such as microangiopathy and macroangiopathy (Erciyas et al. 2004). Hyperglycemia condition resulted in elevated levels of reactive oxygen species, via metal-catalyzed autoxidation or enzymatic oxidative reactions of glucose (Martin-Gallan et al. 2003). Altered energy metabolism, changes in antioxidant defense systems, and local tissue damage are among other suggested causes of oxidative stress (Seghrouchni et al. 2002).

The elevated oxidative stress was resulted from the increased levels of free radicals due to increase in production and/or decrease in the antioxidant defense system (Lyons 1992). The increase in oxidative stress together with poor glycemic control could enhance the damage of the biological macromolecules such as protein, lipid and DNA, which was also associated with the aetiology of diabetic complications (Blasiak et al. 2004). Oxidative products in plasma and cell membrane could be used as the main markers and as an index of in vivo oxidative damage in diabetes mellitus (Lyons 1991; Armstrong & Al-Wadi 1991). A broad range of oxidative products has been described (Aruoma et al. 1989), including lipid peroxides, which are also the precursor to the other reactive intermediates, such as alkoxy radicals, and hydroxyalkenals formed in lipid peroxidation reaction including MDA and 4-HNE. In this study, higher levels of MDA and 4-HNE, resulted from lipid peroxidation process were seen in the STZ-induced rats, which could be due to the poor glycemic control and high production of free radicals.

MDA and 4-HNE are known to interact with DNA (Vaca et al. 1988). Such interactions can cause DNA damage, which can lead to cytotoxicity and genotoxicity (Esterbauer et al. 1991). Recently, significant increase in the levels of 8-oxo-deoxyguanosine, a marker for oxidative damage, has been reported in the lymphocytes of diabetics (Dandona et al. 1996). As cellular enzymes efficiently repair DNA damage, its measurement gives a snapshot view of the level of oxidative stress, in contrast to measurement of oxidation of other biomolecules, which are not repaired and/or have a slow turnover, such as lipids or proteins. DNA oxidation may therefore be of considerable value in following the progress of the disease and its metabolism (Collins et al. 1998).

Conflicting reports regarding levels of DNA damage in mononuclear cells from diabetic subjects have been reported recently. Collins et al. (1998) reported elevated levels of damaged DNA in mononuclear cells from IDDM patients with poor glycemic control (11.0 ± 2.9 % HbA_{1c}). Levels of damaged DNA are measured using the Alkaline

Comet Assay with an additional modification, which incorporated a digestion of nucleoid DNA with specific endonucleases to enable the detection of specific types of damage such as oxidized pyrimidine and purine. Lorenzi et al. (1987) also found elevated levels of damaged DNA by using the alkali-unwinding assay, in mononuclear cells from patients with poor glycemic control (12.9 ± 2.4 %HbA_{1c}). They also indicated no significant changes in DNA damages in subjects with good glycemic control. In this study, the basal levels of DNA damage were measured in freshly isolated lymphocyte from STZ-diabetic rats by using the Alkaline Comet Assay. There was significantly increased levels of DNA damage of the lymphocyte fraction as compared to controls. This finding is in agreement with previous report that showed an increase in the extent of DNA damage in peripheral lymphocyte from diabetic animals (Imaeda et al. 2002).

As glycemic control plays an important role in the progression and development of secondary complications of DM, the changes in the index levels of DNA damage in mononuclear cells in this study could be associated with high levels of glycemic status in streptozotocin-induced diabetic rats. Research carried out by Blasiak et al. (2004) noted not only the elevated levels of DNA damage but also increased susceptibility to mutagens and the decreased efficacy of DNA repair in peripheral lymphocyte of type 2 diabetes mellitus patients. The slowness of DNA repair in diabetic lymphocyte may reflect the overall poor antioxidant protection, which may take part in the detoxification of mutagen such as hydrogen peroxide (Lorenzi et al. 1987).

It would be of interest to know whether clinical intervention to control blood glucose in IDDM patients could also lead to a decrease in oxidative DNA damage. Diabetic patients may have reduced antioxidant defenses, such as diminished activity of glutathione peroxidase, catalase and superoxide dismutase as well as decreased levels of antioxidant (Balsiak et al. 2004). Further investigations are needed, whether the high level of oxidative stress in diabetics can be alleviated by dietary intervention with antioxidants such as vitamin C, vitamin E or carotenoids. The presence of antioxidant including ascorbate, vitamin E and glutathion could possibly have a great influence towards the reaction of the cells to external mutagens. Therefore the aspect of antioxidant protection should take into account as free radical scavenger that may protect the oxidative DNA damage in diabetes mellitus.

While diabetes is associated with a high incidence of cardiovascular complication, its relationship with the elevated risk of cancer is still not clear. From the evidence presented, there was also high level of DNA damage in lymphocytes. However, damage in lymphocyte does not necessarily reflect damage in other cells types that give rise to tumors. Furthermore, the evidence for endogenously produced reactive oxygen species as an initiator of carcinogenesis is still circumstantial (Collin et al. 1998)

In conclusion, this study provides evidence of oxidative damage in diabetic-streptozotocin rats as evaluated using the Alkaline Comet Assay. Our finding showed increased levels of glycemic status and lipid peroxidation products from diabetes rats, which also supported the hypothesis that enhanced lipid peroxidation could contribute to an increased formation of free radicals in diabetes mellitus. Therefore oxidative damage in lymphocytes should be regarded as one of the important biomarkers in oxidative stress.

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TABLE 1. Plasma glucose and HbA1c, MDA and 4-HNE levels in the non-diabetic and diabetic rats after 8 weeks of study

Parameter	NDM	Diabetic
FBG (mmol/l)	6.90 ± 0.22	31.22 ± 0.15 *
HbA1c %	4.57 ± 0.28	10.95 ± 0.93 *
MDA x 10^{-2} (μmol/mg protein)	1.45 ± 0.14	2.50 ± 0.10 *
4-HNE x 10^{-2} (μmol/mg protein)	1.65 ± 0.16	2.84 ± 0.35 *

Data are the mean ± SEM. *P<0.05 compared with NDM group

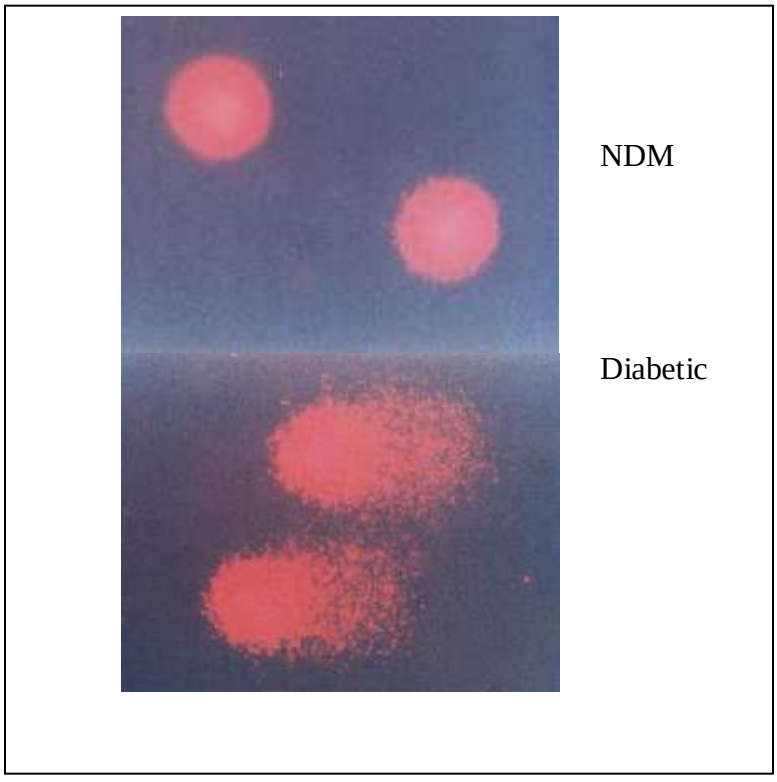

FIGURE 1. Comet Assay images in non-diabetic and diabetic rat

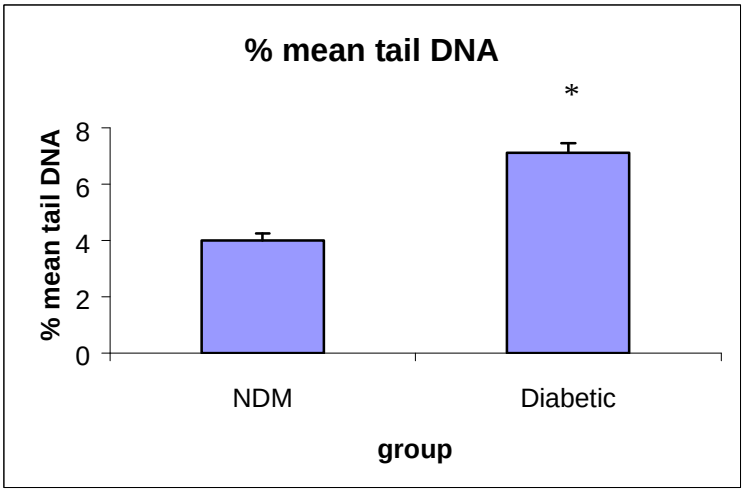

FIGURE 2. Endogenous DNA damage measured by the % of mean tail DNA of peripheral lymphocytes. Data (mean ± SEM) were analyzed by independent t-test. * P<0.05 vs. NDM.

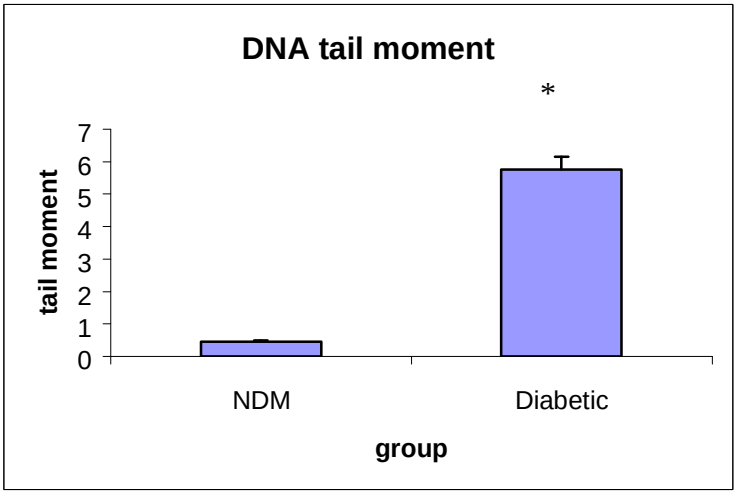

FIGURE 3. Endogenous DNA damage as presented by mean tail moment of peripheral lymphocytes. Data (mean ± SEM) were analyzed by independent t-test. * P<0.05 vs. NDM.