UNIVERSITI PUTRA MALAYSIA

OXIDATIVE DNA DAMAGE IN LEUKOCYTES AND ITS ASSOCIATION WITH METABOLIC CONTROL IN DIABETIC PATIENTS WITH AND WITHOUT MICROALBUMINURIA

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Type 2 diabetes mellitus is the predominant type of diabetes mellitus and the type likely to go undiagnosed. Oxidative damage was suggested in the development of diabetic microangiopathic and macroangiopathic complications in these patients. Since the long-term complications are the main cause of morbidity and mortality in diabetic patients, a quantitative determination of the level of oxidative stress is a valuable indicator of the degree of the severity of the disease and of the effectiveness of treatment. The main aim of this study is to evaluate the extent of oxidative DNA damage with different severity of microalbuminuria in diabetic patients and its associations with other metabolic controls. The comet assay was adopted to measure the level of oxidative DNA damage in leukocytes. One hundred and twenty seven (127) Type 2 diabetic patients with- and without microalbuminuria were recruited from the Outpatients Department and

Medical Outpatients Department, Kajang Hospital. Selection was based on the appearance of urine albumin in the spot urine test by using urine test strip (Micral Test II, Roche). Patients were grouped into four groups, based on the findings in the urine test: N1 (0mg/dl) and N2 (20mg/dl) as Normoalbuminuria, M1 (50mg/dl) and M2 (100mg/dl) as Microalbuminuria. Fasting blood samples were collected for the determination of fasting blood glucose (FBG), glycated haemoglobin (HbA1c), lipoprotein(a), total cholesterol, triglycerides, low density lipoprotein-cholesterol (LDL-Chol) and high density lipoprotein-cholesterol (HDL-Chol), and were done on an automated chemistry analyzer (Cobas Miras, Roche). Low DNA damage (type 0 & 1) was seen in normal subjects and patients with 0mg/dl and 20mg/dl urine microalbumin test. High and complete DNA damage (type 2, 3 & 4) were detected at microalbuminuria marked 50 mg/dl and more. The Microalbuminuria group had shown a significant elevation of DNA damage compared to Normoalbuminuria group (18.54 $\pm$ 3.17% Vs 12.00 $\pm$ 1.37%, $P<0.01$). There was a significant difference in the level of DNA damage between the M2 and the M1 (20.32 $\pm$ 2.82% Vs 16.19 $\pm$ 1.78%, $P<0.01$), and between M1 and N2 (16.19 $\pm$ 1.78% Vs 11.88 $\pm$ 1.46%, $P<0.01$). However, there was no difference in level of DNA damage between the N2 and N1 group. As expected, there were a significant correlation ($p<0.01$) between FBG and HbA1c in both Normo- and Microalbuminuria groups. But no significant difference was detected in the mean values of FBG and HbA1c between all diabetic groups. The level of oxidative DNA damage was increased significantly after the concentration of 20 mg/dl microalbuminuria. Interestingly, the FBG and HbA1c results did not follow with the severity of

microalbuminuria. A significant correlation ($P<0.05$) was detected between the level of DNA damage with FBG and HDL-Chol in both Normo- and Microalbuminuria groups. Concentration of HDL-Chol in Microalbuminuria group (1.06 ± 0.49 mmol/l) was lower compared to Normoalbuminuria group (1.16 ± 0.44 mmol/l). Total cholesterol, triglyceride, LDL-Chol and concentration of Lp(a) did not show any association with the level of oxidative DNA damage. In M2 group, BMI and HbA1c were significantly associated ($p<0.01$ and $p<0.05$ respectively) with the level of DNA damage. In this study, comet assay had shown that cellular DNA damage occurred in the diabetic patients. This study also suggests that oxidative stress occurred in the early stage of complication and even before overt microalbuminuria was detected. Furthermore, FBG and HbA1c did not show any relation with the severity of microalbuminuria indicating that conventional markers do not portray the true condition of the oxidative stress in diabetic patients.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains.

KEROSAKAN DNA SECARA OKSIDATIF PADA LEUKOSIT DAN PERKAITANNYA DENGAN KAWALAN METABOLIK DI KALANGAN PESAKIT KENCING MANIS YANG MEMPUNYAI MIKROALBUMINURIA ATAU SEBALIKNYA

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Type 2 diabetes mellitus merupakan jenis penyakit kencing manis yang utama dan selalunya tidak dikesan awal. Kerosakan oksidatif telah disyaki dalam pembentukan komplikasi mikroangiopati dan makroangiopati di kalangan pesakit kencing manis ini. Memandangkan komplikasi ini dalam jangka panjang merupakan punca utama kepada kecederaan dan kematian pada pesakit kencing manis, maka satu penentuan kuantitatif untuk tahap stres oksidatif ini adalah penting sebagai satu petunjuk kepada tahap keterukan penyakit serta keberkesanan sesuatu rawatan. Tujuan utama kajian ini adalah untuk menilai kerosakan oksidatif pada DNA dengan kepekatan albuminuria yang berbeza pada pesakit kencing manis serta perkaitannya dengan kawalan metabolik yang lain. Assai *Comet* telah digunakan untuk menentukan kerosakan oksidatif DNA pada sel darah putih. Seramai seratus dua puluh tujuh (127) pesakit kencing manis *Type 2* dengan

mikroalbuminuria atau sebaliknya telah diambil dari Jabatan Pesakit Luar, Hospital Kajang. Pemilihan adalah berpandukan kepada kewujudan albuminuria dalam ujian kencing rawak dengan menggunakan *Micral Test II* (Roche). Pesakit telah dibahagikan kepada empat kumpulan berasaskan keputusan ujian kencing: N1 (0mg/dl) dan N2 (20mg/dl) sebagai Normoalbuminuria, M1 (50mg/dl) dan M2 (100mg/dl) sebagai Microalbuminuria. Sampel darah berpuasa telah diambil untuk penentuan glukos darah berpuasa (FBG), *glycated haemoglobin* (HbA1c), *lipoprotein(a),* kolesterol total, triglisarid, lipoprotein-kolesterol ketumpatan rendah (LDL-Chol) dan lipoprotein-kolesterol ketumpatan tinggi (HDL-Chol). Ujian tersebut telah dijalankan dengan menggunakan penganalisa kimia automatik (Cobas Miras, Roche). Kerosakan DNA peringkat rendah (kelas 0 & 1) telah ditemui pada subjek normal serta pesakit dengan mikroalbuminuria berkepekatan 0mg/dl dan 20mg/dl. Kerosakan DNA peringkat tinggi dan sepenuhnya (kelas 2, 3 & 4) telah dikesani pada kepekatan mikroalbuminuria 50 mg/dl dan lebih. Kumpulan Mikroalbuminuria telah menunjukkan peningkatan kerosakan DNA yang signifikan berbanding dengan kumpulan Normoalbuminuria (18.54 $\pm$ 3.17% Vs 12.00 $\pm$ 1.37%, $P<0.01$). Terdapat perbezaan yang signifikan pada tahap kerosakan DNA antara M2 dan M1 (20.32 $\pm$ 2.82% Vs 16.19 $\pm$ 1.78%, $P<0.01$), dan antara M1 dan N2 (16.19 $\pm$ 1.78% Vs 11.88 $\pm$ 1.46%, $P<0.01$). Walau bagaimanapun, tidak ada perbezaan yang signifikan pada tahap kerosakan DNA antara kumpulan N2 dan N1. Seperti yang dijangka, terdapat perkaitan yang signifikan ($p<0.01$) antara FBG dengan HbA1c pada kedua-dua kumpulan Normo- dan Microalbuminuria. Tetapi tidak ada perbezaan yang signifikan dikesan untuk

nilai min FBG dan HbA1c antara semua kumpulan pesakit. Tahap kerosakan DNA oksidatif telah meningkat dengan signifikan pada kepekatan albuminuria 20 mg/dl dan seterusnya. Keputusan FBG dan HbA1c tidak mengikut peringkat kepekatan mikroalbuminuria. Korelasi signifikan ($P<0.05$) telah dikesan antara tahap kerosakan DNA dengan FBG dan HDL-Chol pada kedua-dua Normo- dan Microalbuminuria. Kepekatan HDL-Chol pada kumpulan Microalbuminuria (1.06 ± 0.49 mmol/l) adalah rendah berbanding kumpulan Normoalbuminuria (1.16 ± 0.44 mmol/l). Tahap kolesterol total, triglisarid, LDL-Chol dan kepekatan *Lp(a)* tidak mempamerkan apa-apa perkaitan dengan tahap kerosakan oksidatif DNA. Dalam kumpulan M2, indeks jisim tubuh (BMI) dan HbA1c berkaitan secara signifikan ($p<0.01$ dan $p<0.05$) dengan tahap kerosakan DNA. Dalam kajian ini, assai *Comet* telah menunjukkan kerosakan sel DNA berlaku di kalangan pesakit kencing manis. Kajian juga mencadangkan stres oksidatif berlaku pada peringkat awal komplikasi, dan mungkin sebelum mikroalbuminuria yang jelas dapat dikesan. Malahan, FBG dan HbA1c tidak mempamerkan perkaitannya dengan peringkat kepekatan mikroalbuminuria. Ini menunjukkan petanda-petanda biasa tidak dapat mempamerkan keadaan stres oksidatif yang sebenarnya berlaku pada pesakit kencing manis.

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LIST OF ABBREVIATIONS

DM	Diabetes Mellitus
ROS	Reactive oxidation species
O_2^-	Superoxide Anion
$^{\cdot}OH$	Hydroxyl Radical
H_2O_2	Hydrogen Peroxide
AGE	Advanced Glycosylation End Products
DNA	Deoxyribonucleic Acid
SSB	Single Strand Break
ALS	Alkali Labile Site
FBG	Fasting Blood Glucose
HbA1c	Glycated Haemoglobin
TG	Triglycerides
LDL-Chol	Low Density Lipoprotein Cholesterol
HDL-Chol	High Density Lipoprotein Cholesterol
Lp(a)	Lipoprotein (a)
8-OHdG	8-hydroxy-2-deoxyguanosine
ROOHs	hydroperoxides
TBARS	thiobarbituric acid-reacting substances
SCGE	single cell gel electrophoresis
EtBr	Ethidium Bromide
HCL	Hydrochloric Acid
DMSO	Dimethyl Sulfoxide
NaCl	Sodium Chloride
EDTA.Na2	Disodium Ethylenediamine Tetra Acetate
PBS	Phosphate Buffered Saline
NaOH	Sodium Hydroxide

CHAPTER 1

INTRODUCTION

Diabetes mellitus is a disease characterized by derangement of carbohydrates and lipids. The manifestations of diabetes result from persistently raised blood glucose level and altered energy metabolism as a consequence of reduced production and/or impaired effectiveness of insulin. It is a common cause of blindness, kidney failure, infections necessitating leg amputations, and birth defects. In addition, people with diabetes are twice as likely to develop cardiovascular problems as those without diabetes (Cataldo et al., 1991).

There are generally two forms of diabetes mellitus: Type 1, insulin-dependent diabetes mellitus (IDDM) and Type 2, non-insulin-dependent diabetes mellitus (NIDDM). Both are characterized by a systemic defect in insulin-dependent metabolism but differ in their mechanisms of pathogenesis, insulin deficiency vs. insulin resistance, respectively (Anderson et al., 1998).

Production of reactive oxygen species (ROS) and lipid peroxidation are increased in diabetic patients, especially in those with poor diabetic control and hypertriglyceridaemia (Kitahara et al., 1980). Hyperglycemia, a key biochemical abnormality of diabetes mellitus, not only generates more ROS, but also attenuates antioxidative mechanisms by scavenging enzymes and substances. Therefore, oxidative stress has been considered to be one of the common pathogenic factors of diabetic complications such as cardiovascular disease, nephropathy or neuropathy (Baynes, 1991; Giugliano et al., 1996). In past few years, increased oxidative stress has been indicated by elevated concentrations of lipid peroxidation products such as thiobarbituric acid-reactive substances in the plasma (Lyons, 1991).

Excess ROS not only accelerate oxidative damage to macromolecules such as proteins and lipids, but also to DNA (Dandona et al., 1996). ROS causes base modifications and strand breaks in DNA (Ricther, 1995), and thus result in oxidative damage to DNA. Extended exposure of plasmids to sugars will increase the degree of damage in DNA. Recently, a high concentration of 8-hydroxydeoxyguonosine (8-OhdG), a typical product of DNA oxidation, was reported in the lymphocytes of IDDM and NIDDM patients (Dandona et al, 1996). Guanine residues, deoxyguanosine (dG) were found to be oxidized to 8-oxo-*d*Guo when DNA was exposed to ROS (Richter, 1995). Besides, study by Collins et al. (1998) showed that there was a higher level of DNA damage in lymphocytes from type I diabetic patients compared to healthy

people. Hence, the DNA damage might be useful as a screening marker to indicate and/or predict the development of diabetic microangiopathic and macroangiopathic complications which is caused by oxidative stress.

Problem Statement

Type 2 diabetes mellitus is the predominant type of diabetes mellitus (90 to 95 percent of all cases), and the type likely to go undiagnosed and most often developed in people over 40 and appears to be associated with obesity (often of long duration), abdominal fat, and physical inactivity (Cataldo et al., 1991). Oxidative damage may contribute to the development of diabetic microangiopathic and macroangiopathic complications in these patients. Since the long-term complications are the main cause of morbidity and mortality in diabetic patients, a quantitative determination of the level of oxidative stress is a valuable indicator of the degree of the severity of the disease and of the effectiveness of treatment. There are a number of ways to detect oxidative stress. An alternative method for detecting DNA damage is single cell gel electrophoresis or comet assay. The comet assay is a sensitive and reliable method for measuring DNA single-strand breaks and alkali-labile sites. This assay had been adopted as a useful tool to assess DNA-damage in short-term genotoxicity and human biomonitoring studies (Rojas et al., 1999).

Importance and Validity of Research

In this study, DNA damage will be used as a screening marker in the evaluation of diabetic complications. Although development of oxidative stress in diabetes is well published, the oxidative stress has a primary role in the pathogenesis of diabetic complications is still questionable. It is important to know whether oxidative stress occurs at an early stage in diabetes, preceding the appearance of complications, or whether it is merely a common consequence of the tissue damage, reflecting the presence of complications. In future, the effectiveness of therapy used to treat diabetic patients and its complications can be measured by DNA damage, which is caused by oxidative stress. Hence the failure of therapy for diabetic can be detected and complications can be prevented or slowed down.

This method may be used to monitor levels of DNA damage in individual patients associated with regimen treatment and that the regimen could be changed accordingly. Beside that, the level of DNA damage among some specific groups can be evaluated through this assay and the risk of getting complications can be determined. Also, the comet assay can be used in the study of the mechanism of action of new drugs and in the analysis of interactions between antidiabetic agents acting at DNA level. Such studies may lead to therapeutic approaches for limiting the damage from glycation and oxidation reactions and for complementing existing treatment of the complications of diabetes.

Aim of Study

The main purpose of the study is to compare the extent of oxidative DNA damage with different levels of microalbuminuria in diabetic patients and its associations with other metabolic controls.

Objectives

i) To determine the level of oxidative DNA damage in white blood cells among different groups: Normal group (control group), diabetic patients with- and without microalbuminuria.

ii) To determine metabolic controls (fasting blood glucose, lipoprotein (a) and glycosylated haemoglobin) and lipid profiles (total cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol) that characterize diabetic status among different study groups.

iii) To compare the level of oxidative DNA damage in white blood cells among the study groups.

iv) To compare the measured metabolic controls and lipid profiles among the study groups.

v) To study the associations between the extent of oxidative DNA damage and metabolic controls among the study groups.

vi) To develop comet assay as a simple and sensitive tool for measuring oxidative DNA damage in diabetic cases with different levels of urinary albumin excretion.

Null Hypothesis

1. There is no difference between the level of oxidative DNA damage in white blood cells among the study groups.

2. There is no association between the level of oxidative DNA damage in white blood cells, metabolic controls and lipid profiles among the study groups.

CHAPTER 2

LITERATURE REVIEW

Diabetes Mellitus

The term diabetes mellitus describes a metabolic disorder of multiple etiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both. Hyperglycemia is the cause of the metabolic syndrome and is the parameter most closely monitored to make the diagnosis and to judge therapy (Kaufman and McKee, 1996). Diabetes mellitus may present with characteristic symptoms such as thirst, polyuria, blurring of vision, and weight loss. A diagnosis of diabetes has been defined by the World Health Organization (1985) as either a fasting plasma glucose concentration greater than 7.8mmol/l or a 2-hour postprandial value greater than 11.1 mmol/l.

The cause of diabetes mellitus is unknown. Many etiologic factors are suspected, with major differences between those factors that are etiologic for Type 1 and Type 2 diabetes. Etiologic factors of Type 1 diabetes are