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RESEARCH

DNA fragmentation damage as a predictive marker for diabetic nephropathy in Type II diabetes mellitus

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Background: Increased production of free radicals and oxidative stress in type II diabetic patients could be one of the probable causes for development of complications. The authors hypothesise that such a mechanism also contributes to the development of diabetic nephropathy in those patients.
Aim: The aim of this study was to evaluate the association of DNA fragmentation damage with diabetic nephropathy in type II diabetes mellitus, so as to use it as a future novel predictive marker.
Patients and methods: The study population included 100 patients with diabetic nephropathy, 100 diabetic patients without nephropathy and 100 healthy volunteers as controls. Lipid profile, fasting and post-prandial blood glucose, micro-albuminuria (micro-alb) and glycosylated haemoglobin (HbA1c) were assessed in patients and controls. The technique of capillary electrophoresis was used to detect DNA damage.
Results: The frequency of DNA damage in peripheral blood mononuclear cells was 71% in diabetic nephropathy compared with 45% in non-nephropathy patients ($p < 0.001$). None of healthy controls showed such a finding. Oxidative DNA fragmentation in the diabetic nephropathy group was 3.06 times that in the non-nephropathy group. Neither poor glycaemic control nor dyslipidaemia contributed to DNA damage in diabetic patients. Multivariate analysis showed that positive oxidative DNA damage test (OR1.58, $p = 0.02$) and the duration of ongoing DM (OR 1.48, $p = 0.004$) were the only independent factors contributing to the occurrence of diabetic nephropathy.
Conclusion: Type II diabetic patients have more liability to oxidative DNA damage in general with a significantly higher frequency in diabetic nephropathy. DNA fragmentation analysis can be used as a predictive diagnostic biomarker for diabetic nephropathy.

Keywords: complications, diabetes, free radicals, oxidative stress

Introduction

Diabetes mellitus is a real problem that influences the world as a whole and Egypt in particular. The World Health Organization estimated that the world prevalence of DM among adults was 6.4%, affecting 285 million adults in 2010 with an expected rise to 7.7%, affecting 439 million adults, by 2030. Between 2010 and 2030, there will be a 69% increase in numbers of adults with diabetes in developing and 20% increase in developed countries.[1] The current prevalence of diabetes in adults in the region is estimated to be around 9.2%. Of the 34 million people affected by diabetes, nearly 17 million were undiagnosed and therefore, they would be at considerable risk of diabetic complications and poor health outcomes.[2]

Diabetic nephropathy is a major cause of end-stage renal disease (ESRD) worldwide. Prevalence of diabetic nephropathy gradually increased from 8.9% in 1996, to 14.5% in 2001. The mean age of patients with diabetic nephropathy-related ESRD was significantly higher than that of ESRD from other causes. Mortality was also significantly higher in diabetic patients with ESRD.[3] In diabetic nephropathy there is a progressive decline in the glomerular filtration rate, characterised by glomerular hyper-filtration, glomerular and tubular epithelial hypertrophy, increased urinary albumin excretion, increased basement membrane thickness and mesangial expansion with the accumulation of extracellular matrix proteins (ECM).[4] Increased renal advanced glycation end-products (AGE) in diabetic patients have been linked to structural abnormality observed in diabetic nephropathy such as mesangial expansion, glomerular basement membrane thickening and tubular-interstitial fibrosis.[5] Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the terms collectively describing free radicals and other non-radical reactive derivatives, also called oxidants. Biological free radicals are highly unstable molecules that are products of normal cellular metabolism. They have electrons available to react with various organic substrates such as lipids, proteins and deoxyribonucleic acid (DNA). Free radicals are well recognised for playing a dual role as both deleterious and beneficial species, since they can be either harmful or beneficial to living systems.[6]

Numerous experimental evidences have highlighted a direct link between oxidative stress and diabetes through the measurement of oxidative stress biomarkers in both diabetic patients and rodents. A hyperglycaemic state can lead to an increase in the levels of oxidative DNA damage markers such as 8-hydroxy-2′-deoxyguanosine (8-OHdG), 8-oxo-7, 8-dihydro-2′-deoxyguanosine (8-oxodG), lipid-peroxidation products measured as thiobarbituric acid-reactive substances (TBARS), protein oxidation products such as nitrotyrosine and carbonyl. In the meantime they can all lower the activity of antioxidant enzymes.[7,8] Due to the ability of ROS to directly oxidise and damage DNA, proteins and lipids, free radicals are believed to play a key role in the onset and progression of late diabetic complications.[9]

During the last decade, capillary electrophoresis applied to the biological systems is emerging as a new bio-analytical method with the advantages of fast analysis time, automation, on-column injection and detection, reproducible analysis and high resolving power for the separation testing of double-stranded (ds) DNA fragments.[10,11] It is a sensitive, simple, inexpensive and rapid method that can be used to detect DNA damage to the individual cells and reveal the presence of double-strand breaks, single-strand breaks and alkali-labile sites.[11,12]

Therefore, this study was planned to focus on the relationship between frequency occurrence of DNA damage in type II diabetic individuals and the presence of diabetic nephropathy, with assessment of its validity as a possible diagnostic biomarker for detection of diabetic nephropathy.

Patients and methods

This is a cross-sectional study that was carried out in the diabetes outpatient clinic and clinical pathology unit at Suez Canal University, Egypt. The study population consisted of 1 009 consecutive patients with diabetic nephropathy (Group 1), 100 age-matched diabetic patients without nephropathy (Group 2) and 100 healthy volunteers (Group 3) as controls. Data were collected after obtaining informed consent from all subjects using an interview questionnaire. All individuals were subjected to a complete history, physical examination (height, weight and BMI were calculated) and laboratory investigations. Groups 1 and 2 comprised patients known to have type II diabetes mellitus diagnosed according to ADA guidelines.

Diabetic nephropathy was diagnosed if the patient had positive urinary albumin determined by immunoassay using a morning spot urine, urinary albumin/creatinine ratio ranging from 30 to 299 mg/g creatinine in two or more of three specimens or either urinary albumin excretion rate of 30–299 mg/24 hr urine collection or 20–199 µg/min in timed urine collection.

Patients with either type I diabetes mellitus who refused to participate, with chronic cardiovascular, lung or liver disease or micro-albuminuria associated with a non-diabetic cause were excluded from the study.

A 10 ml blood specimen was collected from each patient in the first session and 3 ml in the second session as follows. In the first session 4 ml of blood was collected and divided into two EDTA tubes, one for DNA extraction and the other for glycated haemoglobin measurement, while 3 ml of blood was collected after 8 h fasting in a sterile plain tube for measuring of fasting blood sugar; 3.0 ml of blood was collected after 12 h fasting in a sterile plain tube for measuring triglycerides, cholesterol and high-density lipoprotein. In the second session 3 ml of blood was collected (2 h post-prandial) in a sterile plain tube for measuring post-prandial blood sugar. A spot urine sample was taken for micro-albumin measurement.

Lipid profile, micro-albuminuria, FBS, PPS and HbA1c were analysed using a Cobas 6000 auto-analyser (Roche Diagnostics GmbH, Mannheim, Germany).

DNA extraction and fragmentation analysis

Genomic DNA was extracted from peripheral blood using avcommercially available spin-column technique kit for DNA extraction (QIAamp®DNA Blood Mini Kit) according to the manufacturer's instructions (QIAGEN, Inc, Hilden, Germany). DNA yield and purity was assessed using a spectro-photometrically Thermo Scientific NanoDrop™ Spectrophotometer (Nanodrop) (Thermo Fisher Scientific Inc, Waltham, MA, USA).

Analysis of DNA degradation was done using QIAxcel DNA Kits for automated analysis of DNA fragments using a QIAxcel capillary electrophoresis instrument that provides robust results for nucleic acid concentrations as low as 0.1 ng/µl and accurate analyses with resolution down to 3–5 bp. After processing, the data are displayed as an electropherogram or gel image.

Results

The study groups were matched for gender distribution. Regarding age, the three groups were divided into 46.41 ± 14.47 years, 52.95 ± 13.25 years and 33.8 ± 10.45 years respectively. The mean body mass index was 31.31 ± 4.57 kg/m^2 in group 1 compared with 30.5 ± 4.78 kg/m^2 and 26.6 ± 5.02 kg/m^2 in groups 2 and 3 respectively (p = 0.008 and 0.009). The mean duration of diabetes was 5.57 ± 2.5 years in group 1 compared with 4.35 ± 1.95 years in group 2 (Table 1).

There was no statistically significant difference regarding level of total cholesterol, triglycerides, LDL-cholesterol, HDL-cholesterol or HbA1c among the diabetic nephropathy and non-nephropathy groups (Table 2). However, there was a statistically significant difference in the mean levels of total cholesterol, triglycerides, LDL-cholesterol and HbA1c among diabetic patients in groups 1 and 2 and the healthy volunteer group 3 (p = 0.0008, 0.0001, 0.001, 0.0001 respectively).

Our study showed that there was DNA damage among patients in group 1 (71.3%), as well as among the patients in the second group (45%) with a statistically significant difference (p < 0.001). Also, there was a statistically significant difference between the two groups regarding quantitation of oxidative DNA damage (3.65 ± 5.8 vs. 0.9 ± 1.3, p = 0.014). The study showed a higher presence of oxidative DNA damage among smokers in comparison with non-smokers (55% vs. 16%). This notification could help to discover other mechanisms that may be incriminated in the pathogenesis of DM complications.

Table 1: Demographic data of studied population

Demographic data		With nephropathy Mean ± SD Median (IQR)	Without nephropathy Mean ± SD Median (IQR)	Control Mean ± SD Median (IQR)
Age		46.41 ± 14.47 48 (22)	52.95 ± 13.25 57 (20)	33.8 ± 10.45 31 (11)
Duration of DM (years)		5.57 ± 2.5 5 (3)	4.35 ± 1.95 4.5 (3)	–
Sex	Male, *n* (%)	24 (24%)	40 (40%)	50 (50%)
	Female, *n* (%)	76 (76%)	60 (60%)	50 (50%)
Smoking*	Negative, *n* (%)	70 (70%)	70 (70%)	100 (100%)
	Positive, *n* (%)	30 (30%)	30 (30%)	0 (0%)
Family history	Negative, *n* (%)	61 (61%)	60 (60%)	90 (90%)
	Positive, *n* (%)	39 (39%)	40 (40%)	10 (10%)

Table 2: Background characteristics of studied population

Lab and exam characteristics		With nephropathy Mean ± SD Median (IQR)	Without nephropathy Mean ± SD Median (IQR)	Control Mean ± SD Median (IQR)
BMI (kg/m^2)		31.31 ± 4.57 30 (4)	30.5 ± 4.78 29.5 (6)	26.6 ± 5.02 27 (4)
Cholesterol (mg/dl)		209.35 ± 8.19 202 (61)	214.65 ± 48.54 208 (46.5)	156.5 ± 20.07 150 (30)
TG (mg/dl)		174 ± 87.45 164 (118)	146.15 ± 68.29 127.5 (90.5)	63.1 ± 20.98 62 (31)
HDL (mg/dl)		45.43 ± 13.69 3 (14)	44.9 ± 12.4 46.5 (19)	54 ± 13.06 50.5 (20)
LDL (mg/dl)		128.65 ± 36.89 123 (51)	140.5 ± 46.34 141 (25)	84.6 ± 26.25 80 (47)
FBS (mg/dl)		191.31 ± 85.33 164 (116)	158.2 ± 65.21 144 (100.5)	87.7 ± 8.92 85 (7)
PP (mg/dl)		298.55 ± 102.74 272 (181)	258.8 ± 107.78 261 (178.5)	121.3 ± 14.94 117.5 (15)
HbA1C (%)		8.96 ± 2.14 8.8 (3.2)	8.74 ± 1.98 8.85 (2.4)	5.24 ± 0.43 5.2 (0.4)
ALB (mg/l)		64.98 ± 39.38 50 (35)	16.2 ± 7.04 16 (10.5)	16.5 ± 6.6 15 (7)
Fundus	Negative, *n* (%)	45 (45%)	35 (35%)	90 (90%)
Exam	Positive, *n* (%)	55 (55%)	65 (65%)	10 (10%)
DNA	Negative, *n* (%)	29 (29%)	55 (55%)	100 (100%)
Fragmentation	Positive, *n* (%)	71 (71%)	45 (45%)	0 (0%)

Multivariate analysis showed that the positive oxidative DNA damage test (OR1.58, $p = 0.02$) and the duration of current DM (OR 1.48, $p = 0.004$) were the only independent factors contributing to the occurrence of diabetic nephropathy. The ROC curve for quantity of DNA fragmentation in relation to diabetic nephropathy was studied. The cut-off point of DNA fragmentation for diagnosis of diabetic nephropathy was 1 strand break with an area under the curve of 0.7466, which provides a sensitivity of 71.43% and a specificity of 70.00%.

Discussion

The present study reported increased oxidative DNA damage in peripheral blood leucocytes of diabetic nephropathy compared with non-nephropathy patients. Moreover, DNA fragmentation was exclusively found in more than two-thirds of diabetic nephropathy patients compared with none of the healthy control group. Previous studies concerning DNA damage and diabetes revealed contradictory results. Several studies showed an increased extent of DNA damage in type II diabetic patients compared with controls.[13] On the other hand, other studies showed a lack of association between diabetes and increased DNA damage levels.[14] The discrepancy between different studies is possibly due to differences in glycaemic control, patient age, and duration of diabetes, treatment methods and techniques used to measure oxidative stress.[13]

Goodarzi *et al.*[12] reported on a significant positive correlation between urinary 8-OHdG, a biomarker of oxidative DNA damage, and both fasting blood glucose and HbA1c. In the present study, there was no correlation between DNA damage and either blood sugar or HbA1c. Theoretically speaking, hyperglycaemia causes glucose auto-oxidation, glycation of proteins, activation of polyol metabolism and subsequent formation of ROS. It has also been demonstrated that hyperglycaemia is associated with an increased production of free radicals in the mitochondria and may contribute to greater DNA damage.[15]

The study did not find any statistically significant difference between duration of diabetes and DNA damage. This has been reported by other authors. In diabetes, Ibarra-Costilla and his colleagues[11] suggested that long-term chronic exposure causes adaptation of response to damage and produces less genetic damage than initial exposure.

One of the complications of diabetes is nephropathy. Diabetic nephropathy is associated with DNA damage that increases with progression of nephropathy. In the current study, there was a statistically significant positive correlation between quantitative DNA damage and severity of micro-albuminuria. Such a finding was also supported by Hinokio *et al.*,[14] who reported a significant positive correlation between 8-oxo, 2′-deoxy-guanosine in urine as a marker for oxidative stress and micro-albuminuria among type II diabetic patients.

In this study, there is no statistically significant correlation between DNA damage and either serum total cholesterol, triglycerides, HDL-cholesterol, LDL-cholesterol or BMI. Other studies have revealed increased DNA damage in obesity with a significant positive correlation with cholesterol, triglycerides and LDL-cholesterol. Also, DNA damage was present in atherosclerotic plaques and in circulating cells of patients with atherosclerosis.[15] It is not known whether DNA damage in diabetes directly

Table 3: Background characteristics of DNA fragmentation in positive and negative diabetics

Patient characteristics		DNA fragmentation		*p*-value
		Negative	Positive	
		Mean ± SD	Mean ± SD	
		Median (IQR)	Median (IQR)	
Age (years)		51.08 ± 14.02	46.73 ± 14.44	0.233
		54 (18)	46.5 (22)	
Duration (years)		5.08 ± 2	5.3 ± 2.63	0.845
		5 (2)	5 (4)	
BMI (kg/m^2)		31.24 ± 5.26	30.98 ± 4.27	0.866
		31 (7)	30 (4.5)	
Sex	Male, *n* (%)	20 (20%)	34 (34%)	0.215
	Female, *n* (%)	80 (80%)	66 (66%)	
Family	Negative, *n* (%)	52 (52%)	65 (65%)	0.255
History	Positive, *n* (%)	48 (48%)	35 (34%)	
Smoking	Negative, *n* (%)	84 (84%)	62 (62%)	0.05
	Positive, *n* (%)	16 (16%)	38 (38%)	
Cholesterol (mg/dl)		212.64 ± 37.23	209.89 ± 53.53	0.649
		203 (46)	202 (59)	
TG (mg/dl)		172.76 ± 67.12	162.16 ± 91.1	0.289
		166 (90)	139 (157)	
HDL (mg/dl)		43.2 ± 10.47	46.45 ± 14.56	0.198
		39 (10)	46.5 (16.5)	
LDL (mg/dl)		132.04 ± 30.17	132.11 ± 44.79	0.97
		134 (29)	128 (44.5)	
FBS (mg/dl)		168.4 ± 69.96	189.27 ± 86.46	0.372
		138 (75)	167.5 (115.5)	
PP (mg/dl)		281.72 ± 109.38	290.05 ± 103.61	0.658
		260 (160)	269 (161)	
HbA1C (%)		8.7 ± 1.88	9 ± 2.21	0.649
		8.5 (2.6)	8.95 (3.3)	
ALB (mg/l)		37.64 ± 25.21	58.34 ± 45.01	0.056
		40 (27)	44 (37.5)	
Fundus	Negative, *n* (%)	32 (32%)	47 (47%)	0.2
Exam	Positive, *n* (%)	68 (68%)	53 (53%)	

Mann–Whitney test used for quantitative data and chi-square test for qualitative data.

promotes atherosclerosis, or is a by-product of dyslipidaemia of diabetes.

Conclusion

Type II diabetic patients have more liability to oxidative DNA damage in general with a significantly higher frequency in diabetic nephropathy compared with non-nephropathy patients. DNA fragmentation analysis can be used as a valuable diagnostic biomarker for diabetic nephropathy.

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