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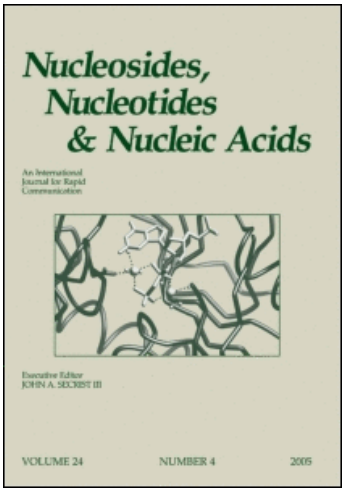

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ASSESSMENT OF CELL-FREE DNA WITH MICROVASCULAR COMPLICATION OF TYPE II DIABETES MELLITUS, USING PCR AND ELISA

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□ *In order to assess the potential biochemical markers in the development, diagnosis, and prognosis of diabetic patient with microvascular complication represented with retinopathy, we analyzed the levels of cell-free DNA by two different techniques. The levels of cell-free GAPDH assayed by quantitative PCR were significantly higher in the plasma samples of diabetic patients with and without diabetic retinopathy than in those of the control group; thus, it is a better biomarker than nucleosomes assayed by ELISA in patients with type 2 diabetes for the early detection of development of microvasuclar complications as retinopathy.*

Keywords Nucleosomes; cell-free DNA; type 2 diabetes mellitus; microvascular complications

INTRDUCTION

Type 2 diabetes mellitus is one of the most common metabolic diseases, affecting about 3% of the human population.[1] Clinically, it is a heterogenous disease, characterized by metabolic disorders, defective blood glucose control, and is frequently associated with chronic complications such as lens cataracts, nephropathy, neuropathy, and cardiovascular diseases.[2]

Hyperglycemia itself often presents in diabetic patients and causes the autoxidation of glucose, glycation of proteins, and activation polyol metabolism. These changes accelerate the generation of reactive oxygen species (ROS). ROS occurring in vivo can cause oxidative damage of amino acids, lipids, proteins, and DNA, therefore, inducing cell death by overwhelming cellular scavenger system.[2] Excess ROS may induce the formation of oxidative DNA damage, DNA strand breaks, base modification, and

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chromosomal aberrations.[3] Furthermore, oxidative stress has been thought to play an important role in the progression of diabetes and its complications.[4]

Multiple studies focusing on DNA-releasing events have provided evidence that cell-free DNA stems from lymphocytes and other nucleated cells in the blood.[5] Apoptotic and necrotic cell death is the source of cell-free DNA, including both histone-bound molecules (such as nucleosomes) and unbound molecules.[6,7]

Supportive proteins—nucleosomes—are more persistent than their unbound counterparts.[8] However, Cell-free total DNA, has the advantage of potentially providing cues for many diseases because of its disease related fluctuations.[9]

In this study, the levels of plasma glyceraldehyde 3-phophate dehydrogenase (GAPDH), representing total (bound and unbound) cell-free DNA, were measured by real-time quantitative PCR, and the levels of nucleosomes, representing bound cell free DNA, were examined by enzyme-linked immunosorbent assay (ELISA). We analyzed the correlation between nucleosome amounts and cell-free GAPDH concentrations in plasma in order to find out whether the presence of circulating cell-free DNA is related to the development of diabetic microvascular complications (retinopathy) as a fresh consideration in pathways involved in hyperglycaemia induced diabetic complications.

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MATERIAL AND METHODS

This study was carried out in the Medical Biochemistry, Internal Medicine, and Clinical Pathology Departments of the Faculty of Medicine at Zagazig University, Egypt. The study was conducted on 70 subjects, which were divided into three groups.

Group 1 was the control group and was comprised of 20 subjects (10 males and 10 females), who were comparable for age and sex with diabetic groups (47–62 mean age 53.2 ± 5.3). Group 2 was comprised of 25 diabetic patients without microvascular complications, but with type 2 diabetes mellitus (DM; 13 males and 12 females). Members of group 2 had a duration of diabetes between 2 and 4 years; their ages were between 42 and 67 years old (mean values ± SD 53.7 ± 7.4); and they reported no history nor showed current evidence of microvascular complications. Group 3 was made up of 25 type 2 DM patients with microvascular complications expressed with diabetic retinopathy (12 males and 13 females); duration of diabetes was between 11 and 20 years and their ages ranged from 40 to 65 (mean value ± SD of 50.7 ± 7.3).

Subjects with retinopathy referred to those with self-reported visual impairment documented by fundus examination. Patients were randomly

recruited from those attending the diabetes outpatients' clinic of Zagazig University Hospitals. All subjects were informed about the purpose and procedures of the study.Informed consent forms were signed by all subjects.

Patients were excluded from the study if they were suffering from liver or preexisting renal disease, strokes, chronic diseases, underlying malignancy, or systemic lupus erythematosus.

All patients and control subjects were submitted to full clinical assessment including a complete medical history and thorough clinical examination, as well as the following routine laboratory investigations, including fasting and 2-hour postprandial blood glucose level; liver functions; blood urea and creatinine; HbA1C; lipid profile including TG; total cholesterol, LDL-C, HDL-C; specific investigations for cell-free DNA and nucleosomes.

Sample Preparation and DNA Extraction

Peripheral venous blood (9 mL) was collected into tubes containing EDTA and processed immediately. Plasma was obtained by a 10 minutes centrifugation at 1600 g and was removed with care to avoid disturbing the underlying buffy coat layer. A further centrifugation step at 16,100 g for 10 minutes was performed to spin down any insoluble remnants in the plasma. Plasma samples were stored at −80°C.

Cell free DNA was extracted from 300 μL of plasma using QIAmp DNA Mini Kit (QIAGEN GmbH, Germany), in a final elution volume of 100 μL, accordingly to the manufacturer's protocol and the purified DNA was stored at −80°C until further use.

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Quantitative Analysis of Cell-Free GAPDH DNA in Plasma Samples

Five micro liters of extracted DNA elution were quantified using quantitative TaqMan real-time PCR analysis (7900, Applied Biosystem, USA), which measures the fluorescence released during the PCR amplification by the two MGB-probes that specifically recognize the chosen nuclear sequences.

The primer pair and VIC-dye-labeled TaqMan MGB probe was used to amplify and quantify the universal GAPDH (GenBank Accession No. J04038):

(forward): 5′ CCC CAC ACA CAT GCA CTT ACG 3′
(reverse): 5′ CCT AGT CCC AGG GCT TTG ATT 3′
(probe): 5′ (MGB)-GTG AAC GTG GAT GAA GTT GG-(VIC) 3′

Our standard TaqMan PCR conditions involve the use of 40 cycles of 15 seconds at 95°C and 1 minute at 60°C according to Zhong et al.[10] The cell-free plasma DNA equivalents were calculated according to highly reproducible standard dilution curves using a known concentration of human genomic DNA ranging from 3.125×10^4 to 10 pg/mL with a dilution factor of 5 (including 31250, 6250, 1250, 250, 50, and 10 pg/mL). Standard curves

with average slopes at approximately −3.3 (−100% efficiency) were obtained using our standard TaqMan PCR conditions. The concentrations of cell-free plasma DNA were expressed as genome equivalents (GE) per mL of plasma. A conversion factor of 6.6 pg of DNA per cell was used for expressing the results as genome equivalents.

Enzyme-Linked Immunosorbent Assay

Mononucleosome and oligonucleosome amounts in plasma were analyzed using a specific Cell Death Detection ELISA Kit, commercially available from Roche Diagnostics GmbH (Penzberg, Germany). Based on the principle of the sandwich immunoassay, single-, and double-stranded DNA, histone-bound DNA and nucleosomes were observable. We used 40 μL of plasma for ELISA analysis. The instruction manual was strictly followed, and samples were assayed as duplicates. Hence, amounts of histone-bound DNA and nucleosomes, represented by their color intensity, were measured. The coefficients of variation were less than 7%.

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Statistical Analysis

The data were analyzed using the SPSS 11 (Statistical Software Package for Windows) software. Data were expressed as mean ± SD and median for quantitative variables. Spearman's rank test was applied to analyze the correlation between levels of circulating cell-free GAPDH DNA and quantities of circulating nucleosomes.

Kruskal-Wallis test used to detect significance between all groups. Man-Whitney test was used to detect significance between groups. $P < 0.05$ was considered significant.

RESULTS

Our results revealed significant increase in cell free DNA measured by PCR in diabetic patients with and without microvascular complication when compared to healthy control (median 10976 and 7890 vs. 4623 GE/ml; Figure 1). By using Kruskal Wallis test to detect significance between various studied groups we found high significant increase of cell free GAPDH in both diabetic groups when compared to control groups ($k = 39.83$ and $P < 0.001$; Table 1).

Measuring nucleosomes by ELISA revealed increase in plasma nucleosomes in diabetic patients with and without microvascular complication when compared to healthy control (median 188 and 181 vs 100 μg/L; Figure 2). By using Kruskal Wallis test to detect significance between various studied groups we found high significant increase of plasma nucleosome in both diabetic groups when compared to control groups ($k = 34.28$ and $P < 0.001$).

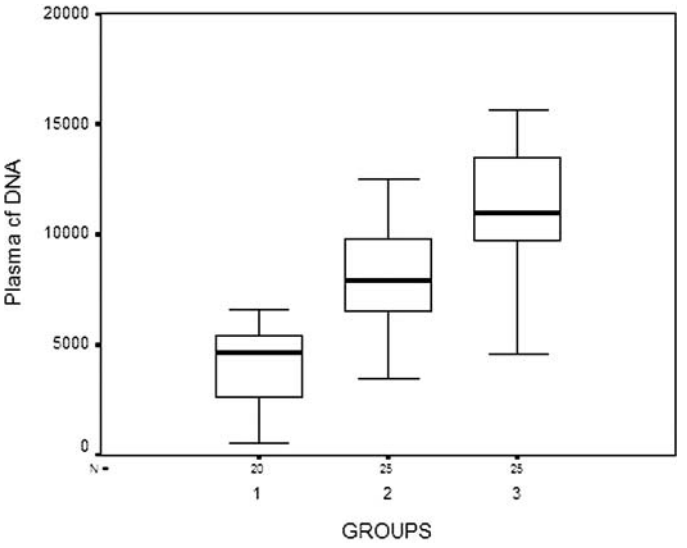

FIGURE 1 Plasma levels of cell-free GAPDH DNA in the 3 study groups. Box plot indicating plasma levels of cell-free DNA (GE/ml) in normal individuals and diabetic patients without and with microvascular complications (retinopathy). The medians are indicated by a line inside each box, the 25th and 75th percentiles by the box limits, and the lower and upper error bars represent the 10th and 90th percentiles, respectively.

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Using Mann-Whitney test to detect significance in between groups, results revealed significant changes of plasma cell free GAPDH and plasma nucleosomes when we compared group 1 and group 3 ($P < 0.001$) and when we compared group 1 and group 2 ($P < 0.001$). On the other hand, when we compared group 2 and group 3 we found significant changes in plasma GAPDH ($P < 0.001$) but no significant change in plasma nucleosomes ($p > 0.5$).

TABLE 1 Quantities of cell-free GAPDH and nucleosomes in plasma of the three studied groups

Group No.	Group 1 20	Group 2 25	Group 3 25	
Age				
Mean ± SD	53.2 ± 5.3	53.7 ± 7.4	50.7 ± 7.3	F = 1.39
Range	47–62	42–67	40–65	P < 0.25
Nucleosome (μg/L)				
Median	100	181	188	K = 34.28
Range	45–146	78–225	115–18	P < 0.001*
Cf-DNA (GE/ml)				
Median	4623	7890	10976	K = 39.83
Range	589–6578	3478–12531	4578–15632	P < 0.001*

GE, genome equivalent; CF, cell free; F = one way ANOVA (F test); K = Kruskal-Wallis test.
*P < 0.05 was considered significant.

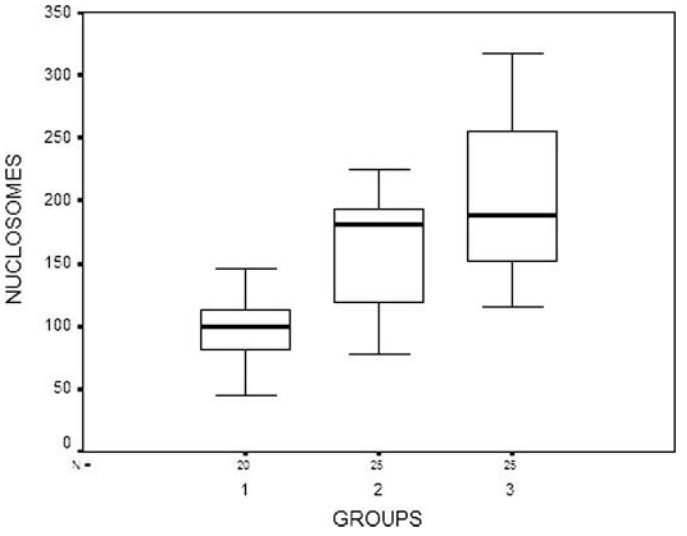

FIGURE 2 Plasma levels of nucleosomes in the 3 study group. Box plot indicating plasma levels of nucleosomes (μg/L) in normal individuals and diabetic patients without and with microvascular complications (retinopathy). The medians are indicated by a line inside each box, the 25th and 75th percentiles by the box limits, and the lower and upper error bars represent the 10th and 90th percentiles, respectively.

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By using Spearman rank correlation, we found that the levels of plasma cell-free GAPDH were correlated with the quantities of plasma nucleosomes ($r = 0.801$, $P < 0.001$).

DISCUSSION

Nucleic acids have been detected in plasma, serum, urine and other body fluids from healthy subjects as well as in patients.[11] With the recent developments in the field of circulating nucleic acids, the application in the diagnosis and monitoring of diseases has increased.[11] Quantitative analysis of circulating cell-free DNA is usually performed by quantitative PCR and ELISA.[6]

Circulating nucleic acids have now been shown to be useful in conditions such as trauma stroke, myocardial infarction and in the oncology field in which detection and monitoring of tumors is now possible by the detection of tumor-derived nucleic acids.[11–13]

In our study, we found a correlation between the quantities of plasma nucleosomes (histone–bound DNA) and the concentrations of cell-free GAPDH (bound and unbound molecules). However, elevated levels of plasma cell-free GAPDH reported difference between complicated and non-complicated diabetic patients, parrallel assessment of plasma nucleosomes did not show clinical significance in detection of microvascular complication.

Nucleosomal complexes consist of duplicate copies of the histones H2A, H2B, H3 and H4 as core proteins, with 146 bp of DNA on the outside.[8] Using agarose gel to analyze the size distribution of cell-free DNA in the circulation, the size fractionation of circulatory DNA indicated that the major portion of circulating cell-free DNA had an approximate molecule size of >0.3 kb (around 78%).[7] Nucleosomal histone bound DNA is considerably smaller than 146 bp and the main part of circulating DNA is larger than 0.3 kb. The ELISA quantifies only the histone bound fraction, which is supposed to be the minor part of circulating cell-free DNA that results mainly in normal cell death.[14]

In agreement with our results, Janusz et al.[15] found that the levels of basal endogenous and oxidative DNA damage in diabetes patients were higher than in control subjects. And that type 2 diabetes mellitus may be associated not only with elevated oxidative DNA damage but also with increased susceptibility to mutagens and the decreased efficacy of DNA repair. Our results were also supported by that obtained by Butt and Swaminathan,[11] who found that in diabetic patients, circulating nucleic acids can be used for the early detection of development of complications such as retinopathy.

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In our study, we noticed that high levels of cell-free total DNA were present not only in the complicated group but also in the diabetic patients without complications but to lesser extent. In agreement with our results Maura et al.,[16] who observed a significant increase in oxidative DNA damage was observed in leucocytes of type 2 diabetics compared to control subjects and that plasma antioxidant capacity is significantly lower only in patients with poor glycaemic control; moreover, oxidative DNA damage is correlated with glycaemic levels of HbA_{1c}.[17]

Many studies tried to explain the underlying mechanism by which cell-free DNA participates in pathogenesis of diabetic complications. It was speculated that, in diabetes, reactive oxygen and nitrogen species occurring in response to hyperglycaemia cause DNA strand-breakage which result in activation of the nuclear enzyme poly (ADP—ribose) polymerase-1 (PARP-1).[18,19]

PARP activation, on one hand, depletes its substrate, DNA, slowing the rate of glycolysis, electron transport and ATP formation. On the other hand, PARP activation results in inhibition of synthesis of GAPDH by poly-ADP-ribosylation. These processes result in acute endothelial dysfunction in diabetic blood vessels, which importantly contributes to the development of various diabetic complications. Furthermore, inhibition of PARP protects against diabetic cardiovascular dysfunction in rodent models of cardiomyopathy, nephropathy, neuropathy, and retinopathy, PARP activation is also present in microvasculatutre of human diabetic subjects.[20] Clinical trials with safe and effective potent PARP inhibitors will help us determine whether PARP represents a future option in the clinical management of diabetes and its complications.[19] Interestingly, it appears that some clinical or

experimental therapeutic interventions, which are known to have some vascular protective effects in diabetes (antioxidant therapies, PRAR agonists, aldose reducatase inhibitors, angiotensin converting enzyme inhibitors, etc.) are able to suppress the activation of PARP in the cardiovascular system.[21–24]

Moreover, it was shown that diabetic patients had increase level of oxidative DNA damage and decreased effectiveness of DNA repair and that these changes may be associated with increased risk of cancer in type 2 DM patients, since DNA damage and repair play a pivotal role in malignant transformation.[25] It was found that glicalzide, an oral hypoglycaemic drug with antioxidant properties, diminished DNA damage induced by free radicals.[25] Our data suggest that quantitative real-time PCR to analyze total circulating cell-free DNA is more efficient and sensitive than ELISA to analyze only histone-bound cell-free DNA. Plasma cell-free GAPHD DNA measured by quantitative PCR can be considered a better marker than nucleosomes measured by ELISA in patients with diabetes mellitus. TaqMan real-time quantitative PCR can be widely applied as a reliable standard to quantify total cell-free DNA in the circulation.

Last, it is noteworthy to mention that, in addition to the four major molecular mechanisms that have been implicated in hyperglycaemia-induced tissue damage: activation of protein kinase-C (PKC) isoforms via de novo synthesis of the lipid second messenger diacylglycerol (DAG), increased hexosamine pathway flux, increased advanced glycation end products (AGE) formation, and increased polyol pathway flux;[26] the plasma cell-free GAPDH DNA measured by quantitative PCR is emerging as a new consideration in the concept of hyperglycemia-induced diabetic complications or at least a biomarker for their early development and may need further study on their application in monitoring cell death events inside the body and their potential to monitor patients during therapy.

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