Effect of Controlling Blood Glucose Level on Apoptosis in Type II Diabetes Mellitus

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Diabetes mellitus (DM) and its complications are complex syndromes representing universal health problems. Egypt ranks ninth among countries with diabetes where the problem is magnified. Researchers have discussed the effect of hyperglycaemia on apoptosis. However, the aims of this study were to assess CD95 positive cells in controlled and uncontrolled type II diabetic patients and to elucidate the effect of controlling blood glucose level on apoptosis. The study was a case control, included controlled type II diabetics and uncontrolled with or without complications) and healthy control groups. Apoptosis was detected by serum DNA fragmentation using ELISA and CD95 positive cells by flow cytometry. There was highly significant increased serum DNA fragmentation and CD95-positive cells in uncontrolled TII DM (complicated and non-complicated) than controlled DM and healthy control groups. In conclusions, poor glycemic control was associated with increased apoptosis.

Diabetes mellitus is a complex syndrome characterised by absolute insulin deficiency or resistance leading to hyperglycaemia, because of inadequate transport of glucose from the vasculature into fat and muscle. Worldwide, the number of diabetic people is growing faster than expected affecting over 246 million individuals. Around 2% of the people with diabetes live in Egypt (International Diabetes Federation, 2008).

Type II Diabetes Mellitus (TII DM) accounts for 90%–95% of the cases, more common in people older than 45 years (Centers for Disease Control and Prevention, 2007). Obesity is a major risk factor for the development of type II diabetes through associated insulin resistance. A diabetic patient exhibit altered glucose, fat and protein metabolism. These metabolic dysfunctions alter the cellular microenvironment in different tissue types, resulting in “diabetic complications” (Ryan et al., 2009). Glucotoxicity and lipotoxicity have both been implicated in β-cell apoptosis. Advanced glycation end-products (AGEs) are believed to exert their toxic effects partly through the generation of reactive oxygen species (ROS) (Brownlee, 2001; Ryan et al., 2009).

Apoptosis is a programmed cell death characterized by morphological changes, such as cellular and nuclear condensation, fragmentation and shrinkage (Islam & Bestka 2003; Laane et al., 2007). Many kinds of stimuli, including cytokines, hormones, infections, drugs and physical force can induce apoptosis (Catalan et al., 2003; Zheng 2001). Moreover, high glucose concentration triggers apoptosis in various cell types, including peripheral blood mononuclear cells (PBMCs) (Sharifi et al., 2007; Boulanger et al., 2004). Fas ligand, TNFα and TRAIL (TNF related apoptosis inducing ligand) are very important death inducing ligands. Fas is a member of the TNF superfamily is implicated in the initiation of apoptotic signalling in many tissues. Fas-mediated apoptosis occurs in nucleated cells (Itoh et al., 1991; Suda et al., 1993).
Fas engagement sequentially activates caspase family proteases to proceed apoptosis, fas is expressed in activated T cells and NK cells, and works as an effector of cytotoxic T cells and NK cells (Suda et al., 1993; Nagata & Goldstein 1995; Enari et al., 1995; Enari et al., 1996; Tanaka et al., 1996).

Apoptosis plays an important role in several diabetic complications. These include apoptosis of neuronal cells in diabetic neuropathy (Li et al., 2002), diabetes-enhanced myocardial apoptosis, which plays a role in cardiac pathogenesis (Cai et al., 2002), and apoptosis of mesangial cells, which occurs in diabetic nephropathy (Makino et al., 2000; Yamagishi et al., 2002a). Diabetes is associated with the production of pro-apoptotic factors, the formation of reactive oxygen species, tumor necrosis factor, and advanced glycation end-products. Reactive oxygen species are potent inducers of apoptosis (Mates & Sanchez, 2000). Tumor necrosis factor can induce apoptosis by binding to the tumor necrosis factor receptor-1, which has a death domain that triggers the initial events in apoptosis, and by stimulating expression of pro-apoptotic genes (Alikhani et al., 2005; Thorburn 2004). Advanced glycation end-products may also promote apoptosis of critical matrix-producing cells through several mechanisms; the direct activation of caspase activity, as well as indirect pathways that increase oxidative stress or the expression of pro-apoptotic genes that regulate apoptosis (Kaji et al., 2003; Kasper et al., 2000; Yamagishi et al., 2002a & b).

National Diabetes Educational Program (NDEP) is one of the CDC programs, which includes more than 200 public and private organizations translates the latest science and spreads the word that diabetes is serious, common, and costly, yet controllable and, for type II DM, preventable. American Diabetic Association reported in its annual report that keeping your blood glucose levels as close to normal as possible can be a lifesaver. Tight glycemic control can prevent or slow the progress of many complications of diabetes, and provides healthy, active life [National Diabetes Educational Program (NDEP), 2008].

The aim of this study was to assess levels of CD95 positive cells in controlled and uncontrolled type II DM patients, to evaluate the effect of controlling blood glucose level on apoptosis in type II DM and to clarify the association between apoptosis and poor glycemic control.

**Materials and Methods**

The study was carried out in Internal Medicine and Medical Microbiology and Immunology Departments, Faculty of Medicine, Zagazig University from October 2008 to December 2009. The University Ethical Committee approved this study.

Study design: a prospective case-control study was conducted on two groups, diabetic and healthy control groups. The diabetic group included 46 non-obese patients with type II diabetes mellitus; they were already on antidiabetic treatment (oral drugs, insulin or both). Diabetes mellitus was diagnosed by clinical examination and laboratory tests. According to WHO recommendation, fasting plasma glucose equals to or > 126 mg/dl (7.0 mmol/l) and 2-hours post-prandial plasma glucose equals to or > 200 mg/dl (11.1 mmol/l) were considered as diabetic [WHO, 2006].

The patients were subdivided into: (1) Sixteen patients uncontrolled DM group, their mean age was 58 ±10.1 years, mean duration of DM was 11.61±4.2 years and median FBS 192 mg/dl, seven patients were males and nine were females. Diabetic complications were excluded in this group by clinical examination and investigations. (2) Sixteen patients with uncontrolled DM group, with macro and microvascular complication in the form of hypertension, ischemic heart disease and diabetic neuropathy. Six cases (37.5%) had hypertension, five cases (31.25%) had ischemic heart disease and five cases (31.25%) had distal symmetrical polyneuropathy. Their mean age was (60 ±10.5) years, mean duration of DM was (11.3±3.1) years and median FBS was 213 mg/dl, six patients were males and 10 were females. (3) Fourteen patients controlled DM group, their mean age was 52.3 ±7.1 years and mean duration of DM was (10.9 ±3.7) years.
and median FBS 99.5 mg/dl, six patients were males and eight were females. The control group included 20 non-diabetic healthy male subjects attending the hospital outpatient clinic, DM was excluded by clinical and laboratory results, their median FBS 95 mg/dl (FBS <110 mg/dl) and mean age was (54.9±5.3) years.

Cases were excluded if they had secondary causes of type II diabetes, exposed to chronic glucocorticoid treatment, being under immunosuppressive drugs or even smokers. Also, patients with tuberculosis, respiratory and renal diseases. Patients with hepatitis viral infection (HBV or HCV) also were excluded from this study. Informed consent was obtained to be included in our study. All subjects had full medical history and general medical examination.

Methods
After at least 12 h fasting, 10 ml of blood was investigated for fasting plasma glucose levels, HbA1c, total cholesterol, HDL-C, LDL-C and triglycerides levels. Complete blood count (total and differential white cell count) was measured by automated cell counter Cell DYN 1700 (Abbott Diagnostics, Massachusetts, United States). Liver & kidney function tests were examined on Synchron CX7 autoanalyser (Beckman Coulter, Fullerton, USA).

Detection of apoptosis by DNA fragmentation in serum was performed using Cell Death Detection ELISA Plus (Roche Applied Sciences, Germany). This is a quantitative sandwich enzyme-immunoassay principle using mouse monoclonal antibodies directed against DNA and histones, respectively. Five ml of venous blood were taken from each patient and control (without anti-coagulant) using vacutainer tubes. Serum was collected after centrifugation at 2,500 rpm for 10 minutes and stored at -20°C.

Separation of peripheral blood mononuclear cells (PBMCs): They were separated by Ficoll-Hypaque (Biotest, Germany) (1.077 gm/ml), density gradient, centrifugation was done using nylon-wool columns to separate T-lymphocyte, that were counted using trypan blue, cell viability was at least 95%.

Detection of (Fas /APO-1) by flow cytometric analysis: This was done using mouse antihuman CD95 conjugated to fluorescin isothiocyanate isomer-1 (FITC) liquid, and the isotype negative control mouse IgG2A (Sero test product, UK). In brief, two tubes were used for each case, one for the control and the other for sample, each of them containing 100 µl of separated cells. Cell suspension was adjusted to 1 x 10^6 /ml in FACs flow (Becton Dickenson FACs). Then 10 µl of the negative control was added to the control tube, 10 µl of antihuman CD95- FITC was added to the sample. The tubes were vortexed, incubated at 4°C for 30 min (protected from light), then washed by adding 2 ml of FACs flow to each tube and centrifuged at 1500 rpm for 5-10 min at 4°C. The supernatant was aspirated leaving 50 µl of liquid (cell pellet). The cell pellet was re-suspended in 500 µl of FACs flow and after brief vortex, the cells were ready for analysis by flow cytometry (Becton Dickenson, USA) with appropriate filter for FITC emit yellow green colour. Samples were gated using forward and side scatter and using dot plot, which displays fluorescence and light scatter along with corresponding quadrant.

Statistics were used in the analysis process. The lower right quadrant values correspond to the percentage of the positive CD95 cells in the samples.

Statistical Analysis
Data were analyzed according to Epi–Info (2005) and SPSS version 10 software computer package. Data were showed as mean ± SD, t- test and ANOVA were used. Non-parametric data was presented as median and range, Kruskal Wallis was used to test comparison between 3 groups and Mann-Whitney test to compare between two groups. Correlation was performed by Spearman rank test. P value < 0.05 was considered statistically significant.

Results
Nineteen males and 27 female diabetic patients were included in this study. There was no significant difference in mean age and mean duration of DM between the studied groups.

Highly significant elevated HbA1c was found in uncontrolled complicated diabetic patients in comparison to controlled DM, uncontrolled non-complicated and healthy controls (P<0.001). FBS levels were highly significantly elevated (P<0.001) in uncontrolled diabetic patients (both complicated & non complicated) when compared to healthy controls and controlled DM groups.

T-cholesterol and LDL-C levels were significantly elevated in uncontrolled complicated group in comparison with the other studied groups. HDL-C was highly
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significantly reduced in uncontrolled diabetic patients (both complicated & non-complicated) when compared to healthy control and controlled DM groups ($P<0.001$).

Apoptosis in diabetic patients was assessed by DNA fragmentation by ELISA and Fas expression (CD95 T cells) on the surface of PBMC by flow cytometry. We found that, DNA fragmentation and Fas expression levels were high significantly elevated ($P<0.001$) in uncontrolled complicated diabetic group than the other studied groups (Fig. 1).

In addition, these levels were significantly higher ($P<0.001$) in uncontrolled non-complicated diabetic group than in healthy controls and controlled DM groups, while there was no significant difference in DNA fragmentation and Fas expression was found between healthy control and controlled DM (table 1).

A significant positive correlation between fasting blood glucose level and both serum DNA fragmentation and Fas expression (CD95) on the surface of PBMC was observed in uncontrolled diabetic patients (both complicated & non-complicated for DNA fragmentation ($r = 0.51, P = 0.004$) and for Fas expression ($r = 0.44, P = 0.01$) (Figure 2). On the other hand, this correlation was not significant in healthy controls DNA fragmentation; $r = 0.25, P = 0.47$) and $r = 0.27, P = 0.45$ for Fas expression (CD95)) and in controlled DM groups ($r = 0.15, P = 0.42$ for DNA fragmentation and $r = 0.18, P = 0.40$ for Fas expression).

![Figure 1. Detection of Fas expression (CD95) in on the surface of PBMC by flowcytometry.](image-url)
Table 1. Demographic and laboratory data of the studied groups including DNA fragmentation in serum and Fas (CD95) expression by flow cytometry

<table>
<thead>
<tr>
<th>Data</th>
<th>Healthy control</th>
<th>Controlled DM</th>
<th>Uncontrolled non complicated DM</th>
<th>Uncontrolled complicated DM</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Mean ± SD) year</td>
<td>54.9±12.3</td>
<td>60±13.1</td>
<td>58±10.1</td>
<td>60±10.5</td>
<td>NS</td>
</tr>
<tr>
<td>Duration of DM (year)</td>
<td>---</td>
<td>10.9±3.7</td>
<td>11.61±4.2</td>
<td>11.3±3.1</td>
<td>NS</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>4.4±0.9(D)</td>
<td>6.0±0.7</td>
<td>7.0±1.3</td>
<td>8.5±1.9(A)</td>
<td>0.000</td>
</tr>
<tr>
<td>FBS (mg/dl) median (range)</td>
<td>95 (83-117)</td>
<td>99.5 (90-111)</td>
<td>192 (B) (145-263)</td>
<td>213 (C) (188-260)</td>
<td>0.000</td>
</tr>
<tr>
<td>T-cholesterol (mg/dl)</td>
<td>130±29</td>
<td>155±23</td>
<td>194±23 (B)</td>
<td>297±25 (A)</td>
<td>0.000</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>59±16</td>
<td>47±9</td>
<td>42±10 (B)</td>
<td>38±13 (C)</td>
<td>0.000</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>108±10</td>
<td>118±9</td>
<td>126±19</td>
<td>154±7.3 (A)</td>
<td>0.02</td>
</tr>
<tr>
<td>triglycerides (mg/dl)</td>
<td>120±11</td>
<td>131±17</td>
<td>141±37</td>
<td>187±16.2</td>
<td>NS</td>
</tr>
<tr>
<td>DNA Median (Range)</td>
<td>0.067 (0.02-0.10)</td>
<td>0.052 (0.04-0.65)</td>
<td>0.29 (B) (0.08-0.56)</td>
<td>0.49 (0.3-0.57 (A))</td>
<td>0.000</td>
</tr>
<tr>
<td>Fas Median (Range)</td>
<td>1.65 (0.28-3.83)</td>
<td>1.71 (0.31-4.10)</td>
<td>6.72 (B) (3.81-9.5)</td>
<td>9.45 (A)</td>
<td>0.000</td>
</tr>
</tbody>
</table>

A: Uncontrolled complicated DM patients were highly significantly different than all other groups.
B: Uncontrolled non-complicated DM patients were highly significantly different than healthy and controlled groups
C: Uncontrolled complicated DM patients were highly significantly different than healthy and controlled DM only
D: Healthy control subjects were highly significantly different than uncontrolled DM.

Figure 2. Scatter plot showing positive correlation between FBS and DNA Fragmentation in uncontrolled diabetic patients.
Discussion

Diabetes mellitus (DM) is a major public health problem; Egypt faces great threats as it ranks ninth among countries with the most number of diabetics. 7.6 million Egyptians will have the disease by 2025 [International Diabetes Federation, 2008]. The problem is magnified especially with bad control of DM and accompanying complications. Many studies have reported the effect of high blood glucose levels on apoptosis, which is important in the pathophysiology of diabetes. However, the influence of controlling this high blood glucose level of T II DM on apoptosis had not been fully studied. Elevated systemic levels of fatty acids in diabetics are significant contributors to the pathophysiological aspects associated with the syndrome. An over accumulation of unoxidized long-chain fatty acids saturates the storage capacity of adipose tissue, resulting in a lipid ‘spill over’ to the liver, muscle, heart, and pancreatic-islets. Such ectopic lipid deposition has deleterious effects as excess lipids are driven into alternative non-oxidative pathways, resulting in reactive lipid moieties formation that promote metabolically cellular dysfunction (lipotoxicity) and programmed cell-death (lipoapoptosis) (Kusminski, et al., 2009).

In this study, elevated HbA1c, FBS, T-cholesterol and LDL-C levels and reduced HDL-C differ greatly among studied groups (uncontrolled T II DM patients with and without complications). Such results suggest that high concentrations of blood glucose lead to increased glycolysis, mitochondrial metabolic activity and subsequently ROS production. At the same time, they block production of NADP, thus attenuate the antioxidant protection of the cell and increase the toxic effect of oxidizing agents. This finding was in agreement with Jeong et al., 2004 in their observations supported by the fact that high rate of substrate influx to mitochondria induces cell sensitivity to oxidant-induced apoptosis. The same results reported by Hasnan et al., (2010) that there was a high significant difference between studied groups in mean FBS, HDL and A1C ($P < 0.001$).

The effect of hyperglycaemia on apoptosis has been explained in different ways by other authors. Moley (2001) showed that hyperglycaemia up-regulates p53 and down regulates the glucose transporters, GLUT 1, 2 and 3, triggering the mitochondrial death cascade pathway. However, Maedler et al., (2001) had the same explanation of our study that glucose-induced apoptosis is due to interaction between the constitutively expressed FasL and the upregulated Fas. Glucose-dependent cleavage of procaspase-3 to activated caspase-3 and glucose-induced DNA fragmentation represent apoptotic β-cell death. In their different studies Allen and his colleagues in 2005 agreed with Gao et al., (2007) and reported that hyperglycaemia induces oxidative and nitrosative stress in many cell types causing the generation of species such as superoxide, nitric oxide and peroxynitrite and their derivatives. They also demonstrated the same concept as our study about the role of high glucose-mediated apoptotic cell death and its relevance to the complications of diabetes such as neuropathy, nephropathy and cardiovascular disease. Massimo et al., (1999) had another explanation in their in vitro study on the effect of high glucose on tissue culture of human pancreatic islets as it modulates the balance of proapoptotic and antiapoptotic Bcl proteins toward apoptosis, thus favouring β-cell death.

Glucotoxicity causes apoptotic programmed cell death, which leads to decreased transcription of the insulin gene, induces caspase-dependent pancreatic cell
apoptosis and suppress cell proliferation (Tanaka et al., 1999; Chiquette & Chilton, 2002; Mehta et al., 2006; Han et al., 2008; Ryan et al., 2009).

The results of this work in assessment of apoptosis by DNA fragmentation by ELISA and (CD95 T cells) Fas expression on the surface of PBMC by flow cytometry revealed high increase in their levels which was more obvious in uncontrolled diabetic patients with complications than non-complicated, that further points to the progression of apoptosis with glucotoxicity in those with complications. These results agreed with Risso et al., 2001, Otton et al., 2004 in their different studies and Ryan et al., (2009) where they studied both T I and T II DM as poorly-controlled diabetic patients presented increased DNA fragmentation as compared with cells obtained from healthy patients and thus explain the impaired immune function in poorly controlled diabetics. However, our aim about the effect of controlling blood glucose level on apoptosis and its hopeful results were discussed and proved by Gao et al., 2007 in their experimental research on rats that good blood glucose control at early stages of TII DM can decrease the number of apoptotic cells in the retina; the decreased apoptosis is correlated with the down-regulation of Bax to Bcl-2 ratio.

In conclusion, our study unveiled that poor glycemic control in T II DM patients especially that accompanied with complications was associated with elevated DNA fragmentation and Fas expression (CD95) levels, while under good glycemic control, the levels of DNA fragmentation and Fas expression (CD95) did not differ from those of healthy control subjects. There was significant positive correlation between fasting blood glucose level and both serum DNA fragmentation and Fas expression (CD95) on the surface of PBMC in uncontrolled DM group and non-significant correlation with healthy control and controlled DM groups.

From our results we recommend that it is mandatory to normalize blood glucose levels in diabetic patients to protect various body cells against exposure to the apoptotic process associated with glucotoxicity as a trial to prevent or minimize the risk for development of diabetic complications that proceed to serious morbidities and even mortality.

References


14. International Diabetes Federation. (2008). Egyptian Foot Care Centre to Improve Diabetes Foot Care with grant from the International Diabetes Federation, Egyptian Foot Care Centre to Improve Diabetes Foot Care with grant from the International Diabetes Federation PRESS RELEASE Brussels/Alexandria – March 13, 2008


