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Original article

HUVECs from newborns with a strong family history of diabetes show increased apoptosis by flow cytometry with annexin V

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ABSTRACT

A family history of type 2 diabetes mellitus (DM) increases the probability to develop DM and endothelial dysfunction. Human umbilical vein endothelial cells (HUVECs) isolated from healthy newborns with familiar background of diverse diseases show early alterations such as less resistance to shear stress. The aim of this study was to evaluate the apoptosis by flow cytometry in HUVECs obtained from healthy newborns with (experimental) and without (control) a strong family history of DM, exposed to different glucose concentrations.

Methods: HUVECs were incubated in M-199 culture media (containing a 5 mmol/L physiological glucose concentration) or supraphysiological glucose concentrations (15 or 30 mmol/L), for 48 h. Apoptosis was quantified by flow cytometry with annexin V and a polycaspase assay kit (FLICA, Immunochemistry Technologies LLC), and cell death was measurement by propidium iodide (PI, St Cruz Biotechnology Inc) positive stain.

Results: Experimental HUVECs showed higher levels of apoptosis in the presence of increasing glucose concentration ($p < 0.01$), whereas control HUVECs showed low levels of apoptosis.

Conclusions: Our results suggest that HUVECs, isolated from healthy newborns with a strong family history of DM, have an abnormal predisposition to apoptosis.

1. Introduction

It is now accepted that most of the so-called chronic degenerative diseases are multifactorial in origin, and the human umbilical vein endothelial cells (HUVECs) study model has showed early alterations for diverse diseases such as systemic lupus erythematosus (SLE) [1], type 1 diabetes mellitus [2], atherosclerosis [3], etc.

Atherosclerosis is a complex disease involved in major fatal events such as myocardial infarction and stroke. It is the result of interactions between metabolic, dietetic and environmental risk factors acting on a genetic background that could result in endothelial susceptibility [4]. Type 2 diabetes mellitus (DM) is a disease of multifactorial origin in which environmental risk factors (sedentary lifestyle, obesity, and dyslipidemia among others) act on a genetic background not well defined to date. A family history of DM increases the probability to develop DM and endothelial dysfunction [5]. High glucose (HG) reduces endothelial cell proliferation with a concomitant increase in apoptosis [6].

Co-treatment with concentrations of methylglyoxal and glucose comparable to those seen in the blood circulation of DM patients (5 μmol/L and 15–30 mmol/L, respectively) could cause cell apoptosis or necrosis in HUVECs in vitro. Co-treatment of HUVECs with 5 μmol/L methylglyoxal and 20 mmol/L glucose significantly increases cytoplasmic free calcium levels, activation of nitric oxide synthase (NOS), caspase-3 and -9, cytochrome *c* release, and apoptotic cell death. In contrast, these apoptotic biochemical changes are not detected in HUVECs treated with 5 μmol/L methylglyoxal and 30 mmol/L glucose, which appeared to undergo necrosis [7].

Endothelial cell damage can explain diverse alterations in chronic diseases [8]. Most studies of proinflammatory factor events [9] in endothelial cells derived from HUVECs have been focused to measure adhesins and chemokines [10–12] but little has been studied with respect to apoptosis.

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Hyperglycemia causes a small but significant increase in the release of lactate by the HUVECs, but does not alter the release of pyruvate and glucose oxidation. Recent studies have attributed this effect of hyperglycemia to increased oxidative stress and intracellular Ca^{2+}, which in part leads to sequential increases in c-Jun NH_2-terminal N-terminal kinase/Stress Activated Protein kinase (JNK/SAPK) activity and caspase-3 [13]. AMP-activated protein kinase (AMPK) could regulate the increase in the generation of nitric oxide (NO) from endothelial cells [14].

We do not know the endothelium behavior of healthy children, but we do know that with a DM family history, endothelial cells respond in a different manner than those of children without this background. Furthermore there is evidence to suggest the existence of different subtypes of endothelial cells functionally different, coexisting within the same blood vessel, as indicated by biochemical markers [15].

The expression of inflammation-related molecules of endothelial cell isolated from healthy neonates with a strong family history of DM has been rarely analyzed. It is possible that the modifications observed in HUVECs, all of them relevant to the atherosclerosis process, may lead to early inflammatory reactions, thus contributing to the premature initiation of atherosclerotic lesions in these children [16].

The aim of this work was to clarify whether HUVECs of healthy babies, but who come from families with a history of high incidence of DM, respond differently to the activity of annexin V, polycaspase assay kit (FLICA, Carboxyfluorescein, Immunochemistry Technologies LLC) and propidium iodide (PI, St Cruz Biotechnology Inc) as markers of apoptosis than HUVECs without these family-inherited background.

2. Subjects, materials and methods

This was a comparative, open, experimental, prospective, transversal study approved by the Institutional Review Board and the Ethics Committee of the Instituto Nacional de Cardiología "Dr. Ignacio Chávez", Mexico City, with the principles laid down in the Declaration of Helsinki.

Universe of study: Pregnant women in the range of 18–35 years of age with and without family history for DM, with at least high school education and who agreed to donate their umbilical cord with informed consent. There was a clinical history for each pregnant woman and her partner. Umbilical cords with and without background for DM were obtained at the time of delivery. The processing of the umbilical cord was done in the first 12 h of collection.

2.1. Selection criteria

After knowing the background history, there were two groups, (a) umbilical cords of newborns of mothers with a family history for DM and (b) umbilical cords with no family history for DM.

Inclusion criteria: (a) *common in both groups*: pregnant women with prenatal care, cord length at least of 15 cm and healthy newborn infants by clinical assessment, (b) *for the umbilical cords with positive background*: a history of at least three relatives with DM, and (c) *for the umbilical cords without antecedents*: no family history of DM.

Exclusion criteria: (a) premature rupture of membranes of more than 3 h, (b) clinical data of infection, and (c) presence of other diseases in the mother before or induced by pregnancy, acute or chronic, as well as taking medication for disease control.

Criteria for elimination: (a) patients who develop gestational diabetes, (b) patients who did not agree to donate the umbilical cord, and (c) patients who were unaware of their family history.

2.2. Description of procedures

2.2.1. Patient recruitment

Pregnant women and their partners were interviewed at the Hospital General "Dr. Manuel Gea González" about the history of DM. At the time of delivery the umbilical cords from both groups (with and without background) were obtained and placed in sterile M-199 (Sigma, St Louis), in addition to fetal bovine serum (FBS) (HyClone).

2.2.2. Isolation and culture of human umbilical vein endothelial cells (HUVECs)

HUVECs were isolated from each individual cord using type II collagenase (0.2 mg/mL, GIBCO/BRL). Non-pooled cells were cultured in T-75 tissue culture flasks (NUNC) in M-199 (Sigma, St Louis) medium, supplemented as previously reported [5]. M-199 culture medium was considered for endothelial cell culture [17]. The formulation of the medium contains 5.5 mM D-glucose, therefore, for practical purposes the results obtained with HUVECs incubated in non-modified M-199 medium are considered basal values. In some experiments, the M-199 medium was complemented with additional glucose in order to reach a final glucose concentration of 15 or 30 mmol/L. Cells were used within passages 4–6 and were identified as endothelial by their morphology and by uniform positive endoglin (CD105) surface antigen (BD Pharmigen) staining.

2.2.3. Cell proliferation and flow cytometry

HUVECs (5×10^3 cells) were cultured for 24 h in 96-multiwell tissue culture plates with 100 μL of non-glucose supplemented M-199 culture medium (without phenol red). After this time, the culture medium was discarded and 100 μL of fresh culture supplemented with 15 or 30 mmol/L glucose-complemented medium (without phenol red) was added to the cultures for a further 48 h. The plates were washed with phosphate buffer saline (PBS) (PBS: 0.01 M $NaH_2PO_4{\cdot}H_2O$, 0.01 M $Na_2HPO_4{\cdot}7H_2O$, 0.15 M NaCl, pH 7.2) and stained with 100 μL from 0.1% crystal violet solution (w/v) (Sigma) in 70% ethanol for 20 min, after which the plates were washed with deionised water and air-dried. The bound dye was dissolved with 100 μL of 10% acetic acid solution. Optical density was measured at 595 nm in a 96-multiwell-plate spectrophotometer (BIO-TEK Instruments, Winooski, USA). Crystal violet stain has allowed us to evaluate HUVECs cell viability as well as cellular proliferation in relation to the culture time [14].

After 48 and 24 h respectively, the cells were processed in flow cytometry. Reagents used for quantitative measurement of fluorescence cell death were: annexin V (St Cruz Biotechnology Inc); FLICA (Carboxyfluorescein, Immunochemistry Technologies LLC) polycaspase assay kit and PI (St Cruz Biotechnology Inc) for the measurement of changes in membrane secondary to activation of cell death. Quantification of apoptosis of the results for each kit used was reported as a percentage of fluorescence according to the criteria of the kit.

Results are shown as mean. The data obtained was analyzed in SPSS v 15 using Wilcoxon's test. The statistical significance was considered with $p < 0.05$.

3. Results

Umbilical cords were obtained from seven newborns offspring of young non-diabetic women (18–26 years old) with a strong DM family history (three or more first degree relatives with the disease) (experimental HUVECs) and from five pregnant women (20–25 years old) without DM family history (control HUVECs). The characteristics of women and their babies are shown in Table 1.

Measurement of cell death by apoptosis with annexin V was performed with flow cytometry. Fig. 1 shows representative

Table 1
Characteristics of pregnant women and their babies.

	Without DM family history	With DM family history
n	5	7
Age (years)	24	23
BMI (kg/m^2)	28	31
Fasting glucose (mg/dL)	73	83
Pregnancies	2	2
Born alive	1	1
Babies		
Weight (g)	3200	3300
Height (cm)	40	45
APGAR	9/9	9/9

BMI: body mass index, DM: type 2 diabetes mellitus.

experiments in the control (A, B and C) and experimental (D, E and F) groups at 5, 15 and 30 mmol/L glucose for 48 h, respectively. In the measurement with annexin V, the kit can distinguish its union with annexin and PI: viable cells, located lower left (annexin V/PI = −/−); early apoptotic cells lower right (annexin V/PI = +/−) cell necrosis (cell death independet of apoptosis) upper left (annexin V/PI = −/+); necrotic and late apoptotic cells (annexin V/PI = +/+) upper right.

In the experiment with annexin V there were significant differences ($p < 0.05$) in viability, apoptosis and necrosis with 5, 15 and 30 mmol/L glucose (Fig. 2A–C). According to the viability (Fig. 2A) at concentrations of 5 and 15 mmol/L it was higher in the control group (66.2 and 66 fluorescence units, FU) than in the experimental group (48 and 47.46 FU) with $p < 0.05$ and a *Z*-test value = −3059 and −2981, respectively.

With the concentration of 30 mmol/L, we found a difference in viability for the experimental group (60.68 FU) with $p < 0.05$ and a *Z*-test value = −3059 (Fig. 2A). In relation to the measurement of apoptosis with annexin V, it was found an increased activity in the experimental group (25.12, 35.86 and 32.84 FU) in the three concentrations of glucose than in the control group (6.3, 7.2 and 2.1 FU), which was significant with $p < 0.05$ (values for the test are shown in the graphs of Fig. 2B). For the measurement of necrosis, there were also significant differences ($p < 0.05$) between both groups (control group = 3.3, 2.1 and 1.5 FU; experimental group = 23, 16.28 and 13.36 FU), being higher in the group with DM history (Fig. 2C).

As a part of the experiment in endothelial cells, we measured the activity of caspases with FLICA (Carboxyfluorescein, Immunochemistry Technologies LLC). Fig. 3 shows representative measurements by flow cytometry, for the control (A–C) and the experimental (D–F) groups, in three glucose concentrations. With annexin V and FLICA we identified four states of the cells: viable cells (FLICA/PI = −/−); early apoptosis (FLICA/PI = +/−), late apoptosis (FLICA/PI = +/+) and necrosis (FLICA/PI = −/+).

In the measurement with FLICA there were significant differences in viability (Fig. 4A) begin higher in the control group (16.31 and 20.46 FU) than in the experimental group (2.95 and 1.66 FU), only with the concentrations of 5 and 15 mmol/L glucose, respectively. There were no significant differences between groups with the 30 mmol/L concentration (Fig. 4A). In early apoptosis (Fig. 4B), the FLICA positive stain was higher in the experimental l group (79.75, 68.6 and 69.5 FU) than in the control group (38.6, 43.03 and 36.92 FU) for the three glucose concentrations ($p < 0.05$). Finally, in the measurement of necrosis (Fig. 4C), no significant differences were found in both groups, according to the three glucose concentrations that were exposed to for 48 h.

4. Discussion

Recent findings indicate that inflammatory processes might start during childhood [16]. In this regard, we have previously showed that HUVECs isolated from healthy newborns to women

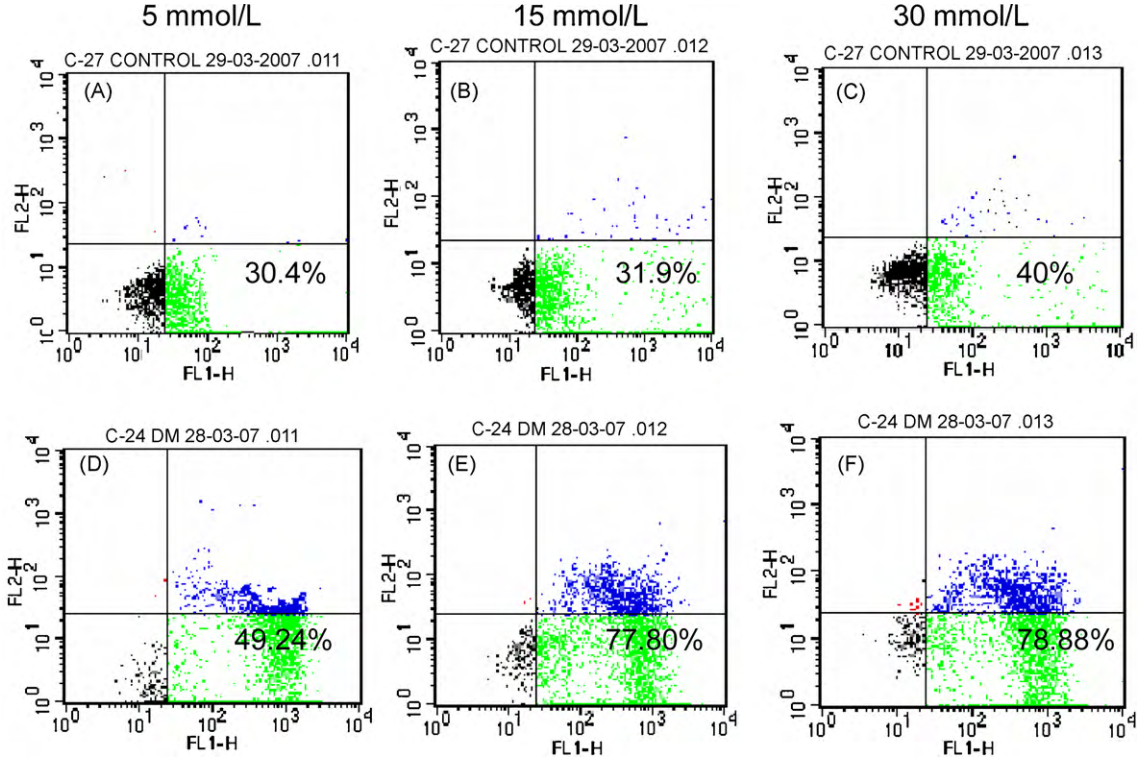

Fig. 1. Measurement of cell death by apoptosis by flow cytometry with annexin V. The control group is represented by A, B and C and the experimental group by D, E and F with 5, 15, and 30 mmol/L glucose for 48 h. The numbers in the lower right quadrant represents the percentage of total fluorescence positive for apoptosis, cells positive for annexin V/PI (+/−). Control: control group, DM: family history of type 2 diabetes mellitus.

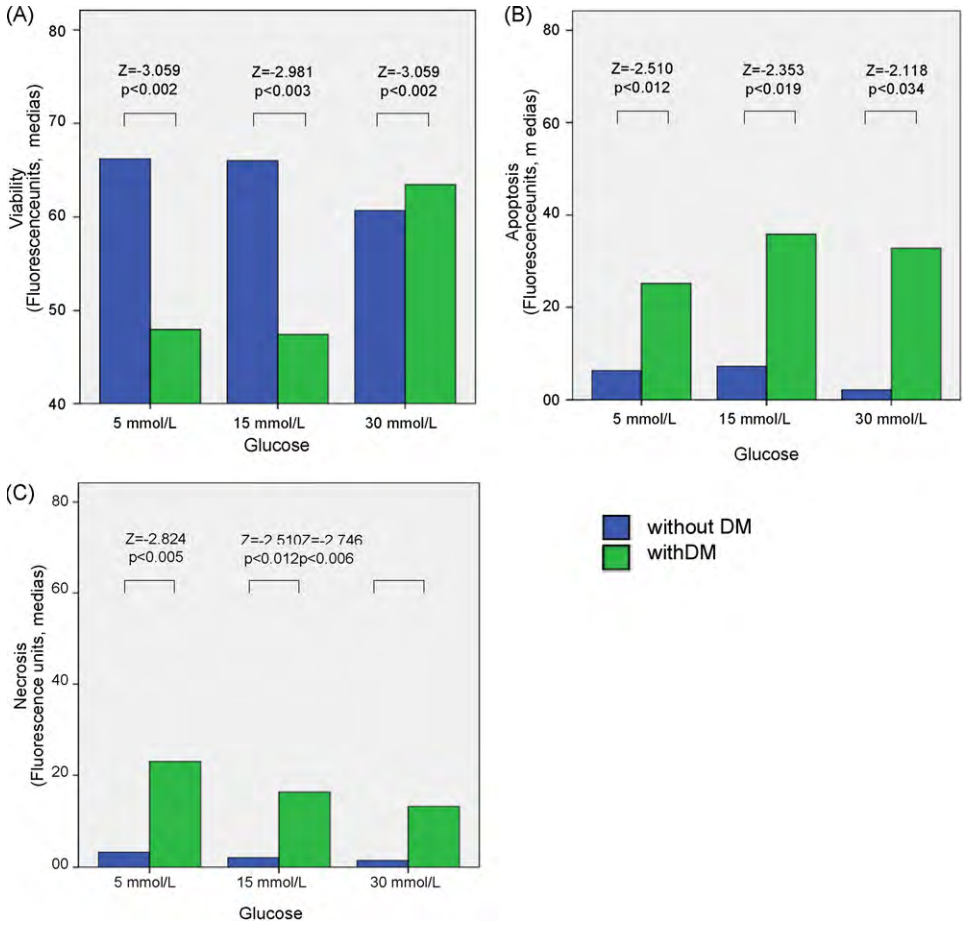

Fig. 2. Measurement of apoptosis with annexin V with 5, 15, and 30 mmol/L glucose. Represents the mean fluorescence units (FU) of viability (A), apoptosis (B) and necrosis (C). DM: family history of type 2 diabetes mellitus.

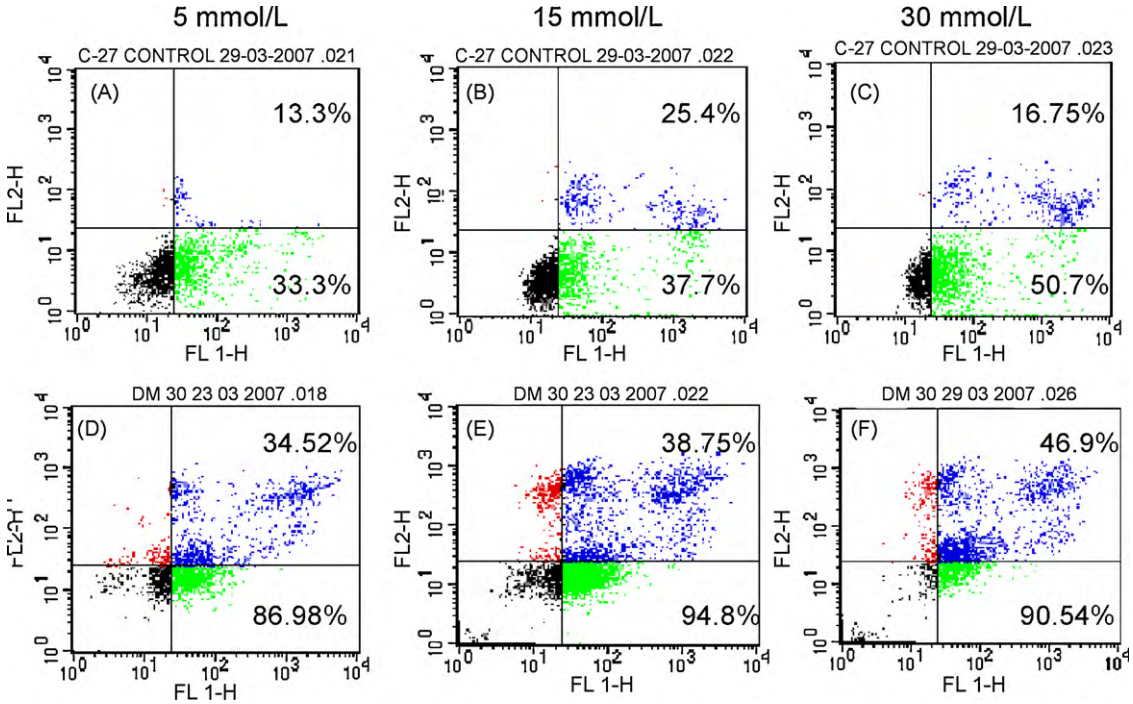

Fig. 3. FLICA measurements of activity for cell death by apoptosis by flow cytometry on endothelial cells. The control group is represented by A, B and C and the experimental group by D, E and F with 5, 15, and 30 mmol/L glucose for 48 h. The numbers in the lower right quadrant represent the percentage of total fluorescence positive for early apoptosis, FLICA/PI (+/-) and the right upper quadrant, total fluorescence positive for late apoptosis, FLICA/PI (+/+). Control: control group, DM: family history of type 2 diabetes mellitus.

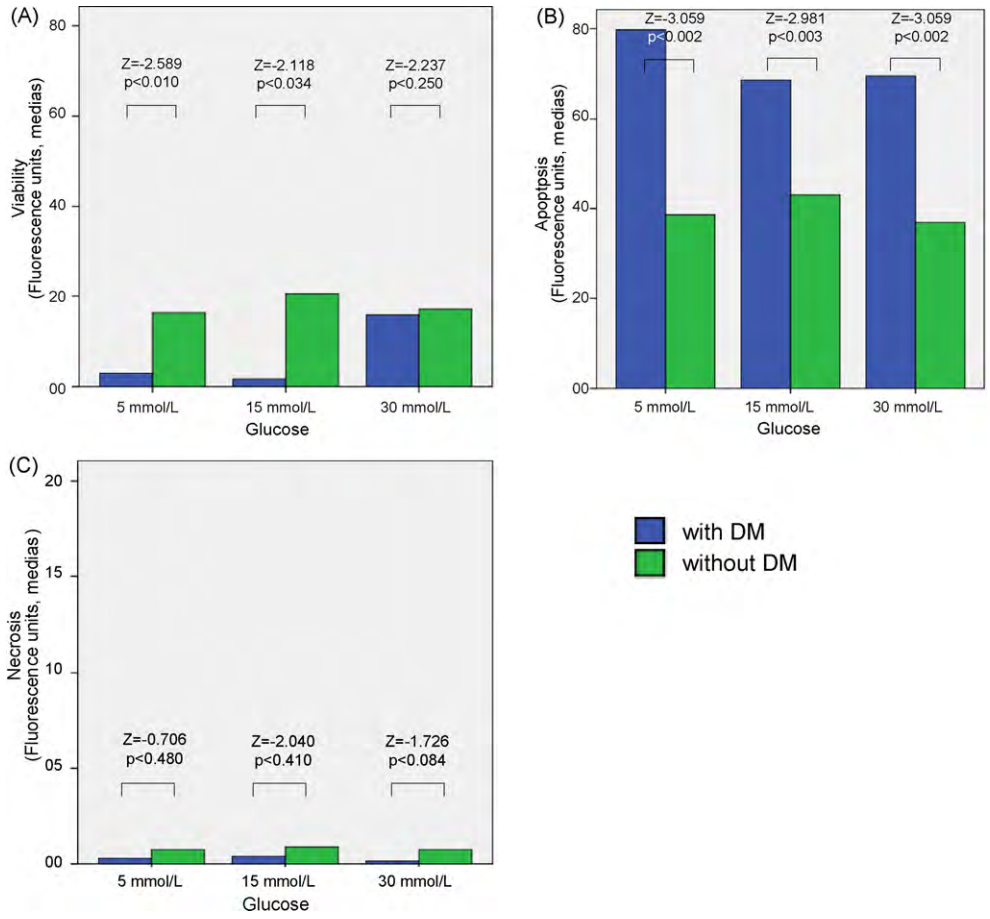

Fig. 4. FLICA measurements of activity to apoptosis in endothelial cells with 5, 15, and 30 mmol/L glucose. Represents the mean fluorescence units (FU) of viability (A), early apoptosis (B), and necrosis (D). DM: family history of type 2 diabetes mellitus.

with inactive SLE show inflammation-related abnormalities that might lead to an early development of SLE in the offsprings [18]. There are also important differences in the labeling of alpha-2,3 or alpha-2,6 sialic acid-containing glycoconjugates, that could lead to a predisposition for early appearance of atherosclerotic lesions [19].

It has been reported that hyperglycemia causes changes in structure and function of the endothelium, such as, reduced or no vasodilation, increased cellular adhesion molecules, decreased intercellular junctions, increased permeability, decreased cell proliferation [20,21] altered cell cycle and increased apoptosis [22]. By the same way, the family history of DM has been associated with alterations in the macrovasculature endothelium such as decrease in the NO production, insulin resistance and early development of atherosclerosis and thus an increased risk of developing cardiovascular disease and DM [23,24].

The fact that hyperglycemia increased apoptosis in endothelial cultures was demonstrated first by Baumgartner-Parzer et al. [25] in HUVECs, and since then by many others [26,27]. It is well established that apoptosis can be induced by various factors such as cytochrome *c*, and other factors released by mitochondria, and this has been commonly associated with a decrease in mitochondrial membrane potential and production of adenosine triphosphate (ATP) [28,29]. Other factors such as caspases (caspase-3, -8, -9), ceramids and oxidative stress, insulin, insulin-like growth factor type 1 (IGF-1) and inactivation of kinases such as Akt (protein kinase B), have also been implicated in the induction of apoptosis in other models [30,31]. Despite this, the metabolic mechanisms by which hyperglycemia initiates apoptosis in the endothelium is not completely understood.

It has been shown previously that incubation of HUVECs for 2 h in a medium containing 30 mmol/L of glucose, does not cause changes in the AMPK activity, malonyl CoA concentration, fatty acid oxidation and released lactate. In contrast, incubation at this concentration of glucose for 24 h results in increased malonyl CoA concentration, decreased fatty acid oxidation and an increase in the incorporation of both radioactive glucose and radioactive palmitate into diacylglycerols.

It has already been reported by other researchers that the concentrations of 15 and 30 mmol/L glucose, may cause increased apoptosis in mixed cultures of endothelial cells, the mechanisms that help to explain this death induced by hyperglycemia have been an increase in oxidative stress, changes in mitochondrial permeability and increased intracellular Ca^{2+} [5,32] that culminate in caspase activation and related intracellular pathways [13,33].

It is interesting that endothelial cells with a family history of DM show poor metabolic response to altered morphology and hyperglycemic stimulus [5]. The results found so far in this investigation show certain trends, which suggest a different response between cells, according to family history of DM. Interestingly, the highest viability was observed in control cells, while the poorest viability was observed with the 30 mmol/L glucose concentration. The mechanism by which a cell responds differently is unknown. In earlier investigations, it has been proposed as explanation an alteration in the mitochondrial response, a change in the glycocalyx, increased glycosylation end products of the pathway and the polyols, which induces changes in the distribution of actin fibers [34], etc.

As already reported [35], apoptosis is increased in endothelial cells and cardiomyocytes in diabetic patients. Of particular interest

is the finding of Frustaci et al. [36] that apoptosis is increased between 61- and 85-fold in endothelial cells and cardiomyocytes, respectively, in ventricular myocardial biopsies from patients with DM history. We found by FLICA that the annexin V activity was increased in cells from healthy newborns with family history of DM, and there was increased apoptosis with higher glucose concentrations. This lets us know that there is a different response between the two groups when exposed to a hyperglycemic stimulus, and by mechanisms that are not yet fully known in case of DM family history there is more susceptibility to apoptosis.

The results obtained so far in this investigation shows that there is an "endothelial programming" in case of DM family history. In fact, HUVECs obtained from healthy newborns with a family history of DM have an innate deficient response to high glucose concentrations [5]. A deficient synthesis of NO may play a role in the early endothelial dysfunction of healthy humans with a strong family history of DM [37]. Our results show how HUVECs, isolated from healthy newborns with a strong family history of DM, have an abnormal predisposition to apoptosis when increasing the glucose concentration.

Understanding the mechanisms involved in endothelial injury will be important in the implementation of prevention and diagnosis, and if possible early treatment to avoid complications in the near future. What is clear until now is that ROS mediate a cellular 'memory' of high glucose stress [38]. These findings comprise physiological mechanisms that could be important in diseases where a proinflammatory state is established such as in gestational diabetic mothers or patients with DM [39].

The fact to understand the mechanisms leading to this disease at an early age will lead in the future more specific preventive strategies, eventually, changing diagnostic criteria, as well as offer new therapeutic alternatives. Ablation of nuclear factor-kappa B (NF-κB) expression by small interference RNA prevents the dysfunction of HUVECs induced by HG [6]. The Akt kinase plays a role in preventing apoptosis in various ways. In particular, its activation is crucial for factors such as mechanical stress (shear stress) [40]. On the other hand it has been seen that AMPK activation might protect against ischemic vascular injury [41]. It has been shown that AMPK phosphorylates (and activates) the eNOS in the same threonine residue than Akt [31] that would lead to the generation of NO. In a more clinical point of view, in vitro data suggest that rosuvastatin has the potential to prevent the damage induced by apoptosis in HUVECs exposed to high glucose concentrations, by reducing oxidative stress [42].

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