Evaluation of BCL-2 and BAX Genes Expression in Hyperglycemia-Induced NIH Cells

Maryam Saleem, Sher Zaman Safi*, Muhammad Imran, Malik Nawaz Shuja, Muhammad Imran

School of Science, University of Management and Technology, Lahore, Pakistan
Department of Microbiology, University of Health Sciences Lahore, Pakistan.

Interdisciplinary Research Centre in Biomedical Materials (IRCBM), COMSATS University Islamabad, Lahore Campus

Department of Microbiology, University of Health Sciences Lahore, Pakistan.

Department of Microbiology, Kohat University of Science & Technology, Kohat-26000 Khyber Pakhtunkhwa, Pakistan

Biochemistry Section, Institute of Chemical Sciences, University of Peshawar, Peshawar, Pakistan

Corresponding Author’s Email: dr.szsafi@gmail.com

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Abstract

The incidence of Diabetes Mellitus (DM) type I and type II is very high all over the world. Excessive glucose levels and failure of one’s body to produce or manage glucose, trigger diabetes. Glucose is known to be responsive to NIH3T3 cells as it alters the expression of a range of genes associated with inflammation and apoptosis. In this study, the toxic effect of glucose was evaluated on NIH3T3 fibroblasts. Cells (NIH3T3) were cultured in media (DMEM), supplemented with 10% FBS and 1% Penicillin-Streptomycin. MTT assay was performed to check the toxic effect of glucose. NIH3T3 cells were treated with high glucose (30mM) for 24 hours. Trizol was used to extract the RNA followed by PCR reactions for gene expression analysis. Glucose treatment for 24 hours, modulated the expression of BCL-2 and BAX genes. The expression of BCL-2 was
reduced while a significant increase was noticed in the expression BAX gene. Our results illustrated that glucose has some toxic effects on NIH3T3 cells. Glucose induces apoptosis by upregulating BAX and down-regulating BCL-2 expressions.

**Key Words:** Gene expression; BCL-2; Apoptosis; BAX; NIH3T3 cells
1. Introduction

Diabetes mellitus (DM) is generally known as a syndrome comprising a range of complications such as hyperglycemia, glucose intolerance, diabetic retinopathy and many other conditions. It is one of the common metabolic diseases with an increasingly growing number of patients that are newly diagnosed [1, 2]. At present, diabetes is placed among one of the well-known non-transmittable infections in the world. In most developed countries, it falls among the 4th–5th foremost reasons for death. It is also widespread in several developing and recently developed nations [3].

Few decades back, T2DM was comparatively uncommon in developing countries; such as, in 1980 the prevalence of the diabetes was <1% in China [4]. The diabetes prevalence and incidence have expanded significantly during the previous two decades in the United States [5]. The actual burden of DM is currently occurring in developing countries, instead developed states. Globally, 80% of the incidents occur in under-developed regions. Asia has arisen as the ‘diabetes focal-point’ worldwide, probably due to urbanization, fast economic progress and nutritional evolution over a moderately short timeframe [4]. Amongst the 10 countries, five are from Asia (India, Pakistan, China, Bangladesh and Indonesia), which are expected to have the highest number of diabetes cases in 2023. The number of young to mid age people with T2DM is higher in developing countries as compared to developed countries [6].

The NIH3T3 cell line is one of the regularly used cell lines in a variety of mechanistic studies. The morphology of these cells are fibroblastic, adherent and are viewed as among the moderately simple to cultivate cell lines. Glucose is toxic for a variety of cell lines including NIH cells [7, 8]. It modulates a number of molecular pathways and consequently alters the expression of various genes including BCL-2 and BAX [9, 10]. Members of BCL-2 family consist of heterodimers or homodimers which are involved in anti-apoptotic, pro-apoptotic and various other cellular activities [10, 11]. When BAX forms a heterodimer with BCL-2, it can act as an apoptotic activator. BAX overexpression stimulates cell death. The heterodimers formation among BAX and death repressor BCL-2 prompts the inhibition of the death-stimulating impacts of BAX [10, 11]. The aim of this study was to evaluate the toxic effect of glucose on NIH3T3 cells and demonstrate the role of BCL-2 and BAX genes.
2. Materials and Methods

2.1 Cell Culture

Normal mouse fibroblast cells NIH/3T3 (ATCC® CRL-1658™) were defrosted at 37°C in a water bath. 1ml of cell suspension was moved to T-75 flask having 15 ml of DMEM media supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin. Cells were subcultured in two T-25 flasks and incubated at 37°C. New media was added after every 2 days until the cells were 80% confluent.

2.2 Treatment of cell culture with Glucose

30mM glucose solution was prepared by adding the right amount of powder of D-Glucose (Molarity weight=198.17) in distilled water, sterilized by autoclaving. This solution was prepared at least one hour before and warmed to 37°C prior to introduction to the cells.

2.3 RNA Extraction

Total RNA was extracted using the Trizol method. To wash the cells, 1ml of PBS was used. This was accompanied by the addition of 1ml Trizol Reagent. Solution was homogenized by pipetting several times. 200 µl of chloroform/1ml Trizol was added to each Eppendorf tube and the tubes were shaken vigorously for 15 seconds and incubated at room temperature for 5 minutes. Tubes were centrifuged at 10,000 rpm for 15 minutes at 4°C. Solution was separated into two phases, and the RNA containing phase was transferred carefully to new Eppendorf tubes. 150 µl of isopropyl alcohol was added to separate aqueous phase, mixed gently and incubated at RT for 10 minutes. Tubes were centrifuged for 10 minutes at 10,000 rpm. This caused the formation of a gel-like pellet. The supernatant was removed and the collected pellet of RNA was washed with 70% ethanol by adding 1 ml of 70% ethanol. Ethanol was discarded and 50 µl of DEPC treated-water was used to dissolve the RNA by gently pipetting numerous times.

2.4 RNA Quantification

The purity and quantity of extracted RNA was calculated by the NanoDrop Spectrophotometer. The absorption by RNA was observed at 260/280 wavelengths while the quantity of RNA was taken in ng/µl.
2.5 cDNA Synthesis

cDNA synthesis (M0253) kit was purchased from NEB. The guideline of the manufacturer was followed to synthesize the cDNA. The items of the kit were defrosted on ice. The contents were mixed by spinning the tubes numerous times. The following components given in Table 1 were mixed followed by for 1 hour 42°C. After that the enzymes were denatured at 80°C for 5 minutes.

Table 1: cDNA synthesis reagents.

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template RNA</td>
<td>2-5 µl depending upon the amount of RNA</td>
</tr>
<tr>
<td>d(T)$_{23}$VN (50 µM)</td>
<td>2 µl</td>
</tr>
<tr>
<td>10X M-MuLV buffer</td>
<td>2 µl</td>
</tr>
<tr>
<td>M-MuLV RT (200 U/µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td>10 mM dNTP</td>
<td>1 µl</td>
</tr>
<tr>
<td>RNase Inhibitor (40 U/µl)</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>Nuclease-free H$_2$O</td>
<td>To a total volume of 20 µl</td>
</tr>
</tbody>
</table>

2.6 Polymerase Chain Reaction (PCR)

Taq DNA Polymerase (M0273) kit was purchased from NEB. The amplification of DNA template was carried out through PCR by following the guideline of manufacturer. All the components were thawed and mixed on ice and immediately transferred to thermocycler preheated at 95°C. (Table 2)

Table 2: PCR Components along with their concentrations and measurements for 25 µl reaction.

<table>
<thead>
<tr>
<th>Component</th>
<th>25 µl reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Standard Taq Reaction Buffer</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>10 mM dNTPs (200 µM)</td>
<td>2 µl</td>
</tr>
<tr>
<td>10 µM Forward Primer (0.2 µM)</td>
<td>1 µl</td>
</tr>
<tr>
<td>10 µM Reverse Primer (0.2 µM)</td>
<td>1 µl</td>
</tr>
<tr>
<td>MgCl$_2$ (2 µM)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Component</td>
<td>Volume</td>
</tr>
<tr>
<td>----------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>Template DNA</td>
<td>3 µl</td>
</tr>
<tr>
<td><em>Taq</em> DNA Polymerase</td>
<td>0.25 µl</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>to 25 µl</td>
</tr>
</tbody>
</table>

**Table 3: Thermocycler conditions.**

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>35 Cycles</td>
<td>95°C, 40-65°C, 72°C</td>
<td>30, 45, 40 seconds</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td>∞</td>
</tr>
</tbody>
</table>

**2.7 Agarose Gel Electrophoresis**

For preparation of 1% agarose gel, 1g of agarose in 100 ml of 1X TAE buffer was mixed and heated for 2 minutes in a microwave oven to completely dissolve the agarose in the buffer. When agarose solution was cooled down to 50°C, 5 µl of ethidium bromide (EtBr) was added to it. This solution of agarose was removed to a gel tray with a well comb placed in it. Comb was removed when the gel was completely solidified. The loading dye (2µl) was added to 15µl of each PCR sample and was observed on UV light.

**2.8 MTT Assay**

NIH cells treated with glucose were cultured in 96 wells plates for 24 hours, having three blank controls with media alone and three simple cells not treated with glucose. Plate was incubated at 37°C for 24 hours. Each well was treated with MTT reagent (10 µl) having a final concentration of 0.5mg/ml and was incubated at 37°C for 4 hours. After 4 hours cells were viewed under microscope, the purple formazan crystals were visible then MTT was removed and detergent reagent (100 µl) was added to all wells and the plate was slightly swirled. The plate was shielded and incubated at RT in dark till the purple crystals were dissolved. The absorbance was calculated in a plate reader at 570 nm.
3. Results

3.1 BCL-2 Expression

Bcl-2 belongs to the family of regulator proteins. In humans it is encoded by the gene BCL-2 which is an oncogene. This protein regulates cell death inducing or inhibiting apoptosis, regulates cell proliferation and angiogenesis. It can support tumor development by both inducing angiogenesis and inhibiting apoptosis. Bcl-2 gene might be either pro-apoptotic or anti-apoptotic. In our study, the expression of Bcl-2 was reduced in glucose treated cells as compared to control (Figure 1)

![PCR results of Bcl-2 gene expression in glucose treated and control fibroblast cells (NIH3T3). (A) Bcl-2 expression is shown in 1.5% gel electrophoresis. (B) Bar chart of band intensity of Bcl-2 expression in NIH cells.](image-url)
3.2 BAX Expression

BAX protein is also known as Bcl-2 like protein as it belongs to the Bcl-2 gene family and functions as an apoptosis regulator protein. BAX- Bcl-2 heterodimer functions as an apoptotic activator. The pro-apoptotic genes comprising BAX, Box and Bak might only take the BH3 domain. The BAX gene is highly prominent in the pro-apoptotic group. BAX overexpression stimulates cell death. According to our results, the expression of Bax is significantly increased in cells treated with glucose as compared to control (Figure 2).
**Figure 2:** PCR results of BAX gene expression in glucose treated and control fibroblast cells (NIH3T3). (A) BAX expression is shown in 1.5% gel electrophoresis. (B) Bar chart of band intensity of BAX expression in control and glucose treated cells

3.3 Cytotoxicity (MTT) Assay

We tested the role of glucose on the viability of NIH cells. It has been observed that glucose does have some toxic effect on NIH cells. In case of control, the number of viable cells were not decreased much as compared to glucose treated. According to our results, glucose treatment reduced the viability of cells in a time dependent manner (Figure 3).

**Figure 3:** Graphical representation of viability of NIH cells after treatment of MTT reagent which clearly shows that glucose reduced the viability of NIH cells.

4. Discussion

Diabetes is one of the widespread lifestyle disorders in the world [12]. The epidemic of diabetes mellitus (DM) and its complications represents a noteworthy health risk globally. Type 2 diabetes
prevalence keeps on increasing with growing numbers of patients at risk of severe diabetes-associated complications.

The aim of this study was to evaluate the genetic expression of apoptosis-associated genes, BCL-2 and BAX in the glucose-induced NIH cells. Furthermore, it also aimed to examine the effect of glucose on the apoptosis (cytotoxicity) of NIH cells. The expression of the pro-apoptotic BAX and anti-apoptotic Bcl-2 genes were studied in order to document their association with hyperglycemia. For this purpose, we took normal mouse fibroblasts (NIH cells) which are well known for the study of angiogenesis, a particular feature of wound healing [13].

We found that hyperglycemia is a main independent risk factor for macrovascular complication of diabetes. It has been observed that hyperglycemia performs a vital role in diabetes advancement and succession alongside other microvascular diabetic problems. Our data demonstrate that high glucose (30 mM glucose) results in NIH cells dysfunction by stimulating the BAX expression and down-regulating Bc1-2 expression. High glucose induced apoptosis has gotten much consideration in current years and there are numerous mechanisms directing the complex signaling pathways that facilitate high glucose induced apoptosis [14, 15, 16]. Moley demonstrated that hyperglycemia up-regulates p53 and down-regulates the transporters of glucose; GLUT1, 2, 3, prompting the mitochondrial death cascade pathway [17, 18]. Along with the oxidative stress, Bax-related events, with consequent caspase initiation promotes the progression of apoptotic cell death in hyperglycemic cells. The hyperglycemic conditions also influences the anti-inflammatory pathways [19]. A study by Gao et al showed that hypoxia stimulates apoptosis and hinders proliferation in the presence of glucose, whereas hyperglycemia considerably constricts the hypoxic-promoted growth response. They reported that high levels of glucose might result in the dysfunction of endothelial cells by downregulating Bc1-2 and triggering the expression of BAX diabetic patients [20]. Another study conducted on mononuclear cells reported that downregulation of bcl-2 results in activation of NF-kB pathway which results in nephropathy in patients of diabetes who have poor glycemic regulation [21]. In cases of diabetic nephropathy, the toxic effect of glucose on myfibroblasts of kidney is well documented [22].

In diabetic complications, a number of genes and molecular pathways are modulated [23-27]. It has been observed that glucose does have some toxic effect on NIH cells. According to our results,
glucose treatment reduced the viability of cells in a time dependent manner. It increases apoptosis through two genes of the Bcl family; anti-apoptotic Bcl-2 gene and proapoptotic BAX gene.

5. Conclusion

This study concludes that glucose inserts some toxic effect on NIH3T3 cells as all glucose-induced cells have shown reduced cell viability. Our results showed that high levels of glucose have led to stimulation of the Bax expression and down-regulation of Bc1-2 expression. Our findings may serve as a base for investigation of mechanisms of delayed or impaired wound healing in various tissues in diabetic patients.

Conflict of Interest

The authors declare no conflict of interest.

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