Scientific Inquiry and Review (SIR)
Volume 5 Issue 2, June 2021
ISSN (P): 2521-2427, ISSN (E): 2521-2435
Journal DOI: https://doi.org/10.32350/sir
Issue DOI: https://doi.org/10.32350/sir/52
Homepage: https://journals.umt.edu.pk/index.php/SIR/Home
Journal QR Code:

Article: **Evaluation of BCL-2 and BAX Gene Expression in Hyperglycemia-induced NIH Cells**

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Article DOI: https://doi.org/10.32350/sir/52.04

QR Code: Maryam Saleem

Citation: Saleem M, Safi SM, Imran M, Shuja MN, Imran M. Evaluation of BCL-2 and BAX Gene Expression in Hyperglycemia-induced NIH Cells. *Sci Inquiry Rev.* 2021;5(2):45–57.

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Indexing

A publication of the
School of Science, University of Management and Technology
Lahore, Pakistan

Evaluation of BCL-2 and BAX Gene Expression in Hyperglycemia-induced NIH Cells

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Abstract

The incidence of Type I and Type II Diabetes Mellitus (DM) is very high all over the world. High glucose levels and failure to manage or produce glucose the body causes diabetes. Glucose is known to be responsive to NIH3T3 cells, since it alters the expression of a range of genes associated with inflammation and apoptosis. In this study, the effect of glucose toxicity was evaluated on NIH3T3 fibroblasts. Cells (NIH3T3) were cultured in media (DMEM) and were supplemented with 10% FBS and 1% Penicillin-Streptomycin. MTT assay was performed to check the effect of glucose toxicity. NIH3T3 cells were treated with high glucose (30mM) for 24 hours. Trizol was used to extract the RNA via PCR 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 of BAX gene. The results illustrated that glucose is toxic to NIH3T3 cells since it induces apoptosis by upregulating BAX and downregulating BCL-2 expression.

Keywords: apoptosis, BAX, BCL-2, gene expression, NIH3T3 cells

Introduction

Diabetes mellitus (DM) is generally known as a disorder comprising a range of complications, such as hyperglycemia, glucose intolerance, diabetic

retinopathy, and many other conditions. It is a common metabolic disease with an increasingly growing number of patients that are newly diagnosed [1, 2]. In most developed countries diabetes is the 4th-5th leading cause of death. It is also widespread in several developing and recently developed nations [3].

A few decades back, Type 2 diabetes (T2DM) was comparatively uncommon in developing countries, such as in the case of China where the prevalence of the diabetes was <1% in 1980 [4]. Diabetes prevalence and incidence has increased significantly during the previous two decades in the United States [5]. Furthermore, developing countries are facing a higher economic burden of DM as compared to developed states. Globally, 80% of diabetes cases occur in under-developed regions. Incidence of diabetes has significantly risen in Asia due to urbanization, fast economic progress, and nutritional evolution over a moderately short timeframe [4]. Amongst the 10 countries expected to have the highest number of diabetes cases in 2023, five are from Asia (India, Pakistan, China, Bangladesh and Indonesia). The number of young to mid age people with T2DM is higher in developing countries as compared to developed countries [6].

NIH3T3 cell line is one of the most regularly used cell lines in a variety of mechanistic studies. The morphology of these cells are fibroblastic and adherent. They are also a relatively simple cell line to culture. 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 such as BCL-2 and BAX [9, 10]. Members of BCL-2 family consist of heterodimers or homodimers which are anti-apoptotic, pro-apoptotic, and are involved in 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. Heterodimer formation in BAX and death repressor BCL-2 inhibits the death-stimulating effects of BAX [10, 11]. This study evaluated the effect of glucose toxicity on NIH3T3 cells to 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 sub-cultured 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. This solution was sterilized by autoclaving. This solution was prepared at least one hour before and warmed to 37°C prior to its 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. This solution was homogenized by pipetting several times. 200 µl of chloroform/1ml Trizol was added to each Eppendorf tube. These 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. The resultant solution was separated into two phases. The RNA containing phase was transferred carefully to the new Eppendorf tubes. 150 µl of isopropyl alcohol was added to the separate aqueous phase, where it was mixed gently and incubated at room temperature for 10 minutes. These 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 using 1 ml of 70% ethanol. The used ethanol was discarded and 50 µl of DEPC treated water was used to dissolve the RNA pellet by gently pipetting numerous times.

2.4. RNA Quantification

NanoDrop Spectrophotometer was used to calculate the purity and quantity of the extracted RNA. The absorption by RNA was observed to be 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. Guidelines given by the manufacturer were 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 components given in Table 1 were mixed for 1 hour at 42°C. After that, the enzymes were denatured at 80°C for 5 minutes.

Table 1. cDNA Synthesis Reagents

Components	**Volume**
Template RNA	2-5 µl depending upon the amount of RNA
$d(T)_{23}VN$ (50 µM)	2 µl
10X M-MuLV buffer	2 µl
M-MuLV RT (200 U/µl)	1 µl
10 mM dNTP	1 µl
RNase Inhibitor (40 U/µl)	0.2 µl
Nuclease-free H_2O	To a total volume of 20 µl

2.6. Polymerase Chain Reaction (PCR)

Taq DNA Polymerase (M0273) kit was purchased from NEB. The amplification of the DNA template was carried out via PCR by following the guidelines provided by the manufacturer. All the components were thawed and mixed on ice. Immediately after, they were transferred to a thermocycler preheated at 95°C. The PCR components and the concentrations of these components used for 25 µl reaction are given in Table 2, while the time and temperature for thermocycler is given in Table 3.

Table 2. PCR Components along with their Concentrations and Measurements for 25 µl Reaction

Component	**25 µl reaction**
10X Standard *Taq* Reaction Buffer	2.5 µl
10 mM dNTPs (200 µM)	2 µl
10 µM Forward Primer (0.2 µM)	1 µl
10 µM Reverse Primer (0.2 µM)	1 µl
$MgCl_2$ (2 µM)	1ul
Template DNA	3 µl
Taq DNA Polymerase	0.25 µl
Nuclease-free water	to 25 µl

Table 3. Thermocycler Conditions

Step	Temperature	Time
Initial Denaturation	95°C	30 seconds
35 Cycles	95°C	30 seconds
	40-65°C	45 seconds
	72°C	40 seconds
Final Extension	72°C	5 minutes
Hold	4°C	∞

2.7. Agarose Gel Electrophoresis

For the 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. It completely dissolved the agarose in the buffer. When the agarose solution's temperature decreased to 50°C, 5 µl of ethidium bromide (EtBr) was added to it. This solution of agarose was shifted to a gel tray with a well comb placed in it. Comb was removed when the gel completely solidified. The loading dye (2µl) was added to 15µl of each PCR sample and was observed under a UV light.

2.8. MTT Assay

NIH cells treated with glucose were cultured in 96 well plates for 24 hours Three well plates were blank controls with media alone, while three well plates containing NIH cells were not treated with glucose The plates were 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, the cells were viewed under microscope. When purple formazan crystals were visible in the cells, then MTT was removed and detergent reagent (100 µl) was added to all wells. The plates were slightly swirled after the detergent reagent was added. The plate was shielded and incubated at room temperature in the dark until the purple crystals dissolved. The absorbance was calculated in a plate reader at 570 nm.

3. Results

3.1. BCL-2 Expression

BCL-2 belongs to a 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 and regulates cell proliferation and angiogenesis. Conversely, it can also 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).

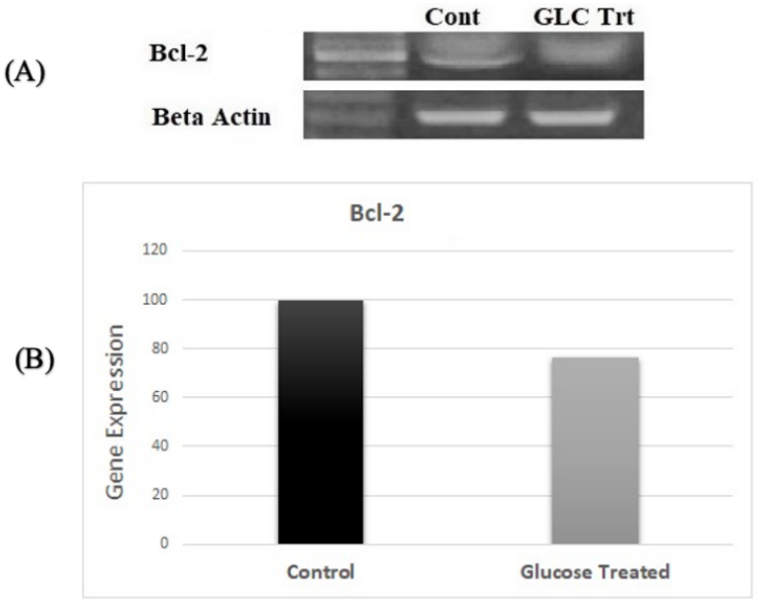

Figure 1. PCR results of BCL-2 gene expression in glucose treated and control fibroblast cells (NIH3T3). (A) BCL-2 expression is shown in 1.5% gel electrophoresis. (B) Bar chart of band intensity of BCL-2 expression in NIH cells

3.2. BAX Expression

BAX protein 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 such as 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).

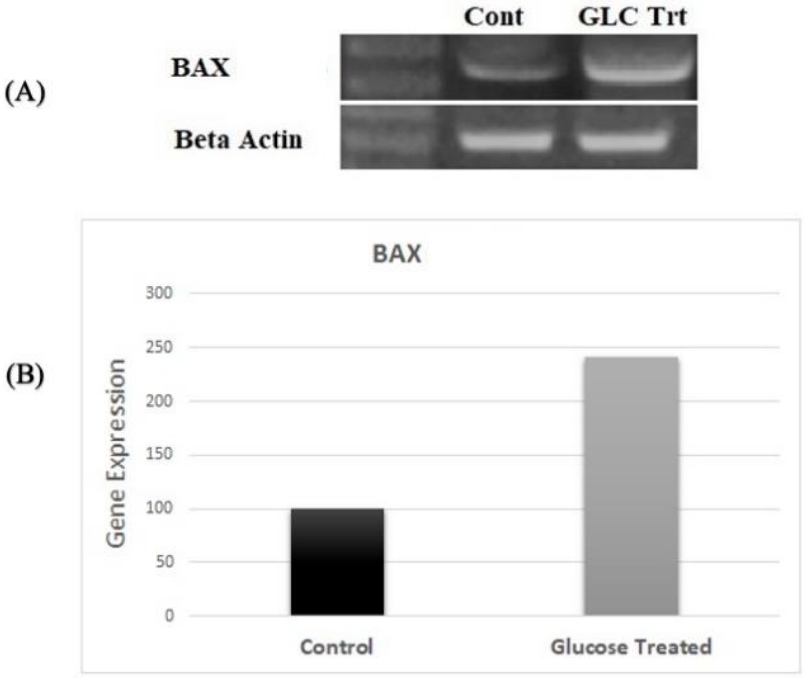

Figure 2. PCR results of BAX gene expression in glucose treated and control fibroblast cells (NIH3T3). (A) BAX expression is shown in 1.5% gel electrophoresis. (B) Bar chart of band intensity of BAX expression in control and glucose treated cells

3.3. Cytotoxicity (MTT) Assay

We tested the role of glucose to check the viability of NIH cells. It was observed that glucose is toxic to NIH cells. The number of viable cells in the control well plates was higher as compared to the well plates with glucose treated cells.

According to our results, glucose treatment reduced the viability of cells in a time-dependent manner (Figure 3).

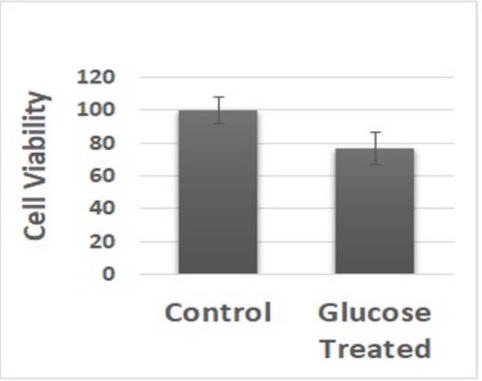

Figure 3. Graphical representation of viability of NIH cells after treatment of MTT reagent which clearly shows that glucose reduced the viability of NIH cells

4. Discussion

Diabetes is one of the most widespread disorder in the world [12]. The epidemic of diabetes mellitus (DM) and its complications represents a noteworthy and global health risk. The high incidence of Type 2 diabetes (T2DM) has led to a significant increase in patients at risk of severe diabetes-associated complications.

This study evaluated the genetic expression of apoptosis-associated genes, BCL-2, and BAX in glucose-induced NIH cells. Furthermore, it also examined 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, Normal mouse fibroblasts (NIH cells) were selected since they are well known for the study of angiogenesis, which is an important feature of wound healing in injured cells and tissues [13].

We found that hyperglycemia is the main independent risk factor for macrovascular complication of diabetes. It was observed that hyperglycemia performs a vital role in inducing diabetes and other microvascular diabetic problems. Our data demonstrated that high glucose (30 mm glucose) causes NIH cells dysfunction by stimulating the BAX expression and down-regulating BCL-2 expression. High glucose-induced apoptosis has received a lot of attention in recent years

There are numerous mechanisms directing the complex signaling pathways that facilitate high glucose-induced apoptosis [14, 15, 16]. Moley demonstrated that hyperglycemia upregulates p53 and downregulates the transporters of glucose, namely GLUT 1, 2, 3, which stimulates the mitochondrial death cascade [17, 18]. Along the oxidative stress, BAX-related events with consequent caspase initiation promotes the progression of apoptotic cell death in hyperglycemic cells. Hyperglycemic conditions also influence 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-prompted growth response [20]. Another study also reported that high levels of glucose downregulates BCL-2 and triggers the expression of BAX to cause a dysfunction of endothelial cells in diabetic patients [21]. Another study conducted on mononuclear cells reported that downregulation of BCL-2 results in the activation of NF-kB pathway, which, in turn, causes nephropathy in diabetes patients who have poor glycemic regulation [22]. The effect of glucose toxicity on kidney fibroblasts is well documented with regard to diabetic nephropathy, [23].

In patients with diabetic complications, a number of genes and molecular pathways are modulated [24-28]. It was observed that glucose is toxic to NIH cells. According to our results, glucose treatment reduced the viability of cells in a time dependent manner. It increased apoptosis through two genes of the BCL family, namely anti-apoptotic BCL-2 gene and proapoptotic BAX gene.

5. Conclusion

This study concludes that glucose is toxic to NIH3T3 cells since all the glucose-induced cells showed reduced cell viability. The results also revealed that high levels of glucose stimulate BAX expression and downregulate BCL-2 expression. These findings can serve as a base for the investigation of delayed or impaired wound healing mechanisms in various tissues in diabetic patients.

Conflict of Interest

The authors declare no conflict of interest.

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