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Original Article

Antioxidant Effect of N-acetyl Cysteine on the Differentiation Improvement of Human Adipose-Derived Stem Cells in a High-Glucose culture

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Abstract

Background: Diabetes is a common disease affecting majority of populations worldwide. Diabetes is characterized by high levels of circulating glucose and leads to most microvascular and macro vascular complications. Bone marrow vascular disruption and increased adiposity are also linked to various complications in type II diabetes mellitus. In addition to these complications, type 2 diabetic patients also have fragile bones caused by faulty mineralization. Diabetic osteopathy is one of the diabetes mellitus complications. N-acetyl cysteine as an antioxidant can improve the differentiation process of mesenchymal stem cells into osteoblasts in a high glucose medium.

Methods: Human adipose-derived stem cells were cultured in different glucose concentrations, and MTT checked their proliferation and survival. Osteogenic differentiation of adipose stem cells was analyzed by examining the expression of *RUNX2* and *osterix* genes by real-time polymerase chain reaction. The alkaline phosphatase expression was analyzed after 14 days of differentiation of these cells. N-acetyl cysteine antioxidant was added to the differentiation medium, and its effect was studied on the Adipose stem cells differentiation into osteoblasts.

Results: the finding of the study show N acetyl cysteine has antioxidant effect on the proliferation, survival, and differentiation of adipose stem cells into osteoblasts in a high glucose medium significantly. N-acetyl cysteine improved osteogenic parameters as *RUNX2, Osterix*, alkaline phosphatase in high glucose culture condition.

Conclusion: Generally, the results of the present study show the protective effects of N-acetyl cysteine on the proliferation, survival, and differentiation of adipose derived stem cells into osteoblasts in a high glucose medium and can be used as an antidiabetic drug in the treatment of osteopathy caused by diabetes.

Keywords: N-acetyl cysteine, ADSCs, Osteoblastic differentiation, High glucose.

Introduction

Diabetes is a chronic disease in which the body cannot use or store glucose. There are two main types of this disease; Type 1 and Type 2. Apart from these two main types,

pre-diabetes and gestational diabetes also exist [1].

The World Health Organization (WHO) predicts that the population of diabetics will increase to 552 million in 2030 [2].

Bone problems such as osteoporosis, one of the complications of diabetes, are public health problems growing with age and population. This disease is a common disorder characterized by a systemic defect in the bone mass and its microstructure, which results in bone fractures [3].

Researchers have shown that high glucose concentration increases cell growth and mineralization and suppresses the expression of several markers with osteoblasts, such as runt-related transcription factor 2 (runx2), collagen type I, osteocalcin, and osteonectin [4].

Moreover, part of the diabetes effects on osteoporosis may be caused by humoral factors. In general, diabetes negatively affects ossification and is associated with an increased risk of osteoporosis and fragility fractures [5].

Chronic high blood sugar in diabetes increases free radicals, which damages the adipose, proteins, and nucleic acids. Oxygen free radicals are molecules with a more significant number of unpaired electrons that are formed in cells [6].

Free radicals are very reactive that combine with any molecule and eventually cause damage to vital macromolecules and cell membranes. Under normal conditions, free radicals are broken by the defense system of antioxidants [7].

Natural antioxidants trap free radicals and prevent oxidative damage. N-acetyl cysteine is an antioxidant supplement converted into an amino acid called cysteine in the body. Cysteine helps produce glutathione, an antioxidant compound that plays an important role in regulating many cellular functions and maintains the immune system in optimal condition [8]. Mesenchymal stem cells are multipotent cells widely used for regenerative and medical purposes. Mesenchymal stem cells are obtained from various body sources that differentiate into cells such as osteocytes, chondrocytes, and adipocytes in *in vitro* conditions [9].

Thus, this research aims to investigate the effect of high glucose medium on the proliferation and differentiation of ADSCs as well as the effect of N-acetyl cysteine (NAC) on mesenchymal cells differentiation into osteocyte cells in a high glucose medium.

Material and Method

Cell culture

Two million human adipose-derived mesenchymal stem cells were purchased from the Research Center of Tissue Engineering Research Center. They were incubated in DMEM/F12 culture medium with 10% FBS supplement at 37 °C and 5% CO2 and 95% air atmosphere. After the third passage, the cells were incubated in different concentrations of glucose (25 µM, 50 µM, and 75 µM) for 72 hours. The control group was considered with 5.5 µM glucose.

3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) assay:

MTT assay was performed to investigate cytotoxicity. To perform this assay, about 5 x 103 cells were cultured in 96-well plates and treated with the desired concentrations of Zingron for 24, 48, and 72 hours, and then the supernatant was removed and 200 µl of 0.05% MTT solution (Sigma-Aldrich) was added to each well. After one hour of incubation at room temperature, the supernatant was removed, and dimethyl sulfoxide (DMSO, Sigma Aldrich) was added to each well to dissolve the formazan crystals. Finally, the optical density (OD) was measured at 570 nm by a spectrophotometer (Thermo Fisher).

ADMSCs differentiation into osteocytes

$5*10^4$ cells were cultured in 24-well plates covered with vitronectin in DMEM/F12 medium and FBS (10%) to differentiate mesenchymal stem cells into osteoblast cells. The cells were cultured with one mM NAC for 72 hours, and then glucose with different concentrations was added to the differentiation medium containing dexamethasone (1 mM), ascorbic acid diphosphate (0.1 M), and glycerol diphosphate (1 M) in the presence of NAC. The cells were cultured in the differentiation medium for 14 days.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA of ADMSCs cells were isolated using TRIzol reagent (Sigma-Aldrich). cDNA synthesis was done using the cDNA reverse transcription kit of Pars Tous company. The qRT-PCR reaction was performed using SYBR Green PCR Master Mix manufactured by Amplicon Cc. The relative expression of genes was calculated using the formula $2^{-\Delta\Delta Ct}$. β-actin gene was used as the internal control.
The genes evaluated in this reaction include: *RUNX2*: F: CCCAGTATGAGAGTAGGTGTCC, R: GGGTAAGACTGGTCATAGGACC. *OSTERIX (OSX):* F*:* ACCCGTTGCCTGCACTCTCF, R: CACAATGTTCTCTCCCCAAGCT, and *β-actin*: F: GGCATCCTCACCCTGAAGTA and R: TGAGTGTAAGGACCCATCGGA as the internal control.

Alkaline phosphatase assay

After 14 days of treating ADMSCs with different glucose concentrations, the cells were washed several times with PBS, lysed by NP4O lysing buffer, and the resultant solution was centrifuged at 12,000 rpm. The supernatant (supernatant solution) was separated. The alkaline phosphatase activity was tested using the alkaline phosphatase kit (Pars Azmoon, Iran) at a wavelength of 450 nm with the colorimetric method and spectrophotometer [11].

Statistical analysis

All experiments were repeated three times. The obtained data in SPSS software version 16 were analyzed as mean ±SD using the ANOVA test ($P<0.05$).

Results

Cell viability in high glucose culture

The results of ADSCs culture in different concentrations of glucose (25 µM, 50 µM, and 75 µM) by MTT assay show that with increasing glucose concentration, the survival rate of the cells decreased so that the lowest cell survival rate is observed at the concentration of 75 µM. Adding 1 µM NAC antioxidant to the cells increased cell survival and did not show a significant difference with the control group (with normal sugar) (Figure 1).

RUNX2 and Osterix genes' expression in the presence of NAC

In the high glucose medium, the *RUNX2* and *OST* genes' expression significantly decreased compared to the control group, so the greatest change was observed in the 75 µM sugar group compared to the control ($P<0.01$). By adding NAC antioxidants, the expression process of osteocyte genes increased and approached the control group (Figure 2).

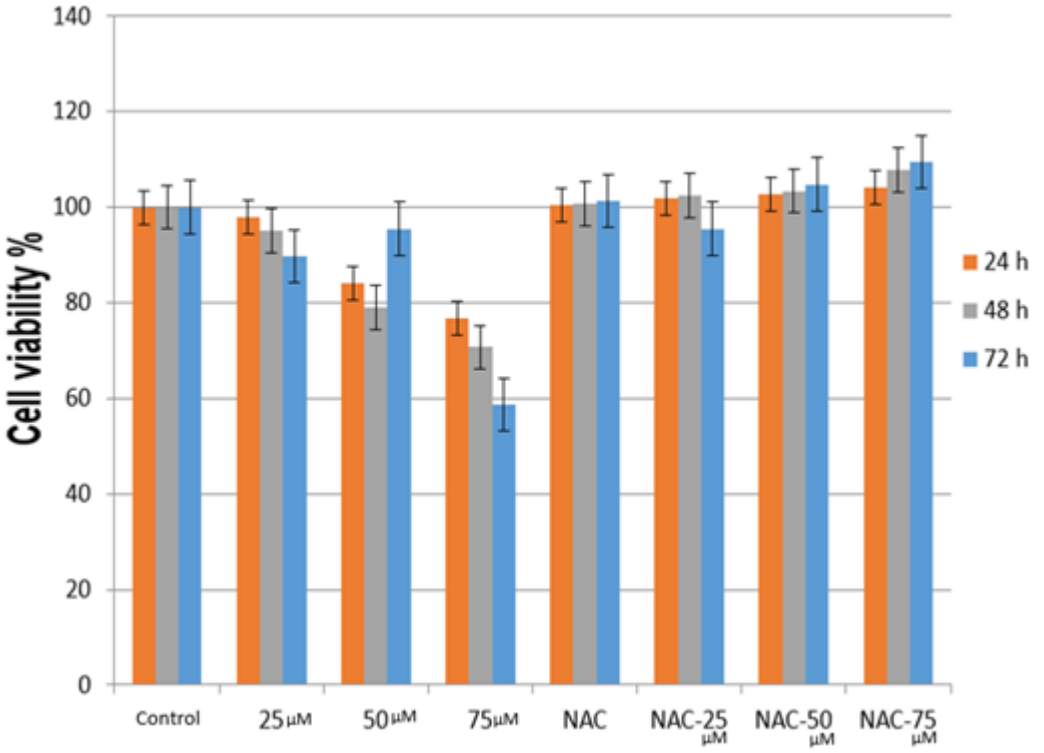

Figure 1: Antioxidant effect of NAC in different glucose concentration of ADMSCs culture. MTT assay revealed NAC effect on different glucose concentration in 24h, 48h and 72h culture

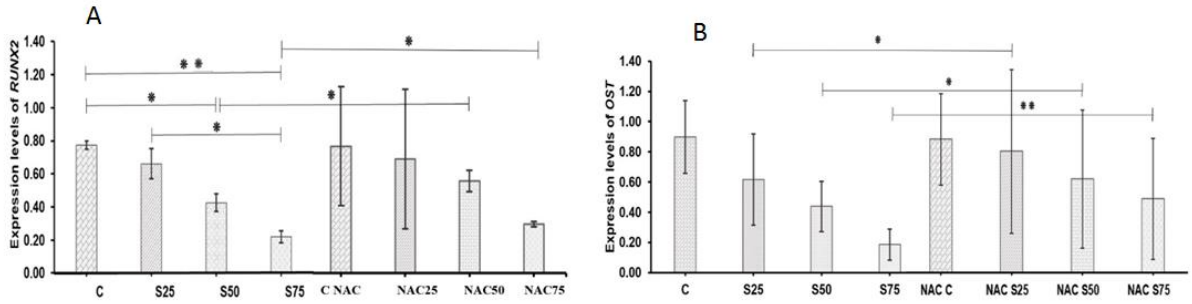

Figure 2: Expression of specialized osteogenic genes in ADSCs at high glucose and NAC. (A) *RUNX2* gene expression in ADSCs in the presence of different glucose concentrations (25, 50, and 75 µM and NAC 1 µM after one week). (B) *OST* gene expression in ADSCs in different concentrations of glucose and NAC (**P<0.05* and ***P<0.01)*

Alkaline phosphatase expression in differentiated osteocyte cells in high glucose medium

After 14 days of culture of mesenchymal stem cells in the differentiation medium, the alkaline phosphatase expression decreased significantly in the concentrations of 50 µM and 75 µM glucose with increasing glucose concentration in the culture medium. Alkaline phosphatase expression increased by adding N-acetylcysteine to the differentiation culture medium and neutralizing the oxidative effect of high glucose (Figure 3).

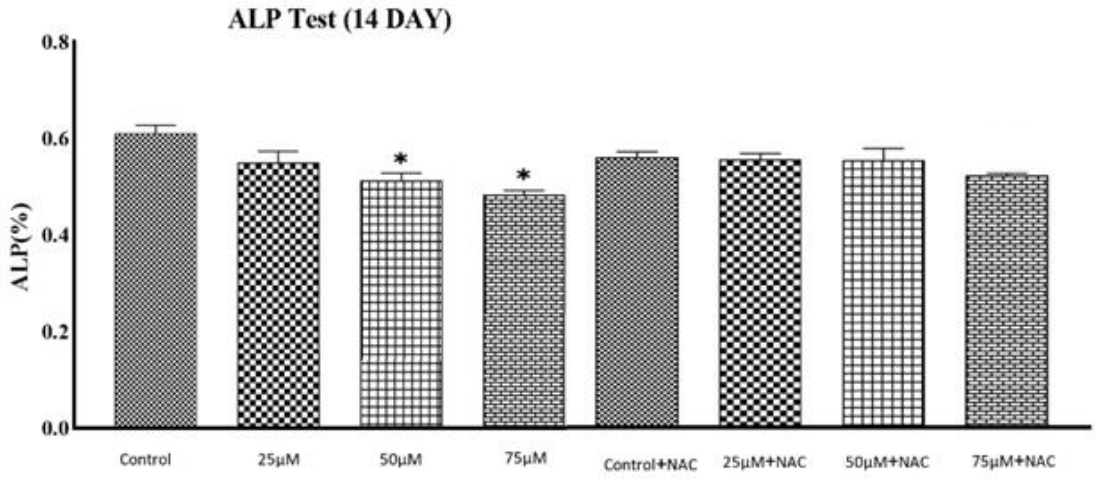

Figure 3: Alkaline phosphatase expression in the osteogenetic medium after 14 days of culture in the presence of different glucose concentrations. The alkaline phosphatase expression decreased significantly in the concentrations of 50 µM and 75 µM glucose ($P<0.05$). Alkaline phosphatase expression increased by adding 1 µM NAC in all glucose concentrations close to the control group (*$P<0.05$)

Discussion

Diabetes mellitus is a metabolic disease known as one of the oldest diseases. Since diabetes mellitus causes many bone diseases such as osteoporosis and fragility due to the long-term exposure to high sugar drugs that reduce blood sugar or its effects can reduce diabetic osteopathy significantly [12].

Proliferation and differentiation of osteogenic cells are keys to the bone health. Hyperglycemia has an adverse impact on the proliferation and differentiation of these cells. Mesenchymal stem cells contribute to bone tissue health by secreting the extracellular matrix and repairing bone damage in bone differentiation [13].

In this study, ADSCs were cultured in different concentrations of glucose to create a hyperglycemic medium, and the effect was investigated on differentiation into osteoblasts. Furthermore, the simultaneous effect of glucose and NAC was studied on the osteogenic differentiation of cells. The results of the present study are consistent with those of previous studies. They show that high glucose harms the differentiation process of stem cells to osteoblasts by inhibiting stem cells' proliferation and differentiation [14].

Moreover, NAC significantly reduces the inhibitory effects of high glucose on stem cell differentiation and proliferation [15].

ALP is a membrane glycoprotein abundantly expressed in the placenta, intestines, kidneys, liver, and bone [16].

The present study evaluated ALP as a marker for the activity of osteoblasts. In addition, genes involved in the osteogenic differentiation of cells such as RUNX2 and osteocalcin were investigated as osteogenic markers. In previous studies, metformin, as an inhibitor of high glucose harmful effects, causes the proliferation and differentiation of osteogenic cells [17].

Although high glucose increases cell survival insignificantly in the first to the third days of cultivation, a significant decrease in cell survival and proliferation was observed from the fourth day. The differentiation process does not occur at high glucose due to the decrease in the speed of metabolic reactions and enzyme activities, and as a result, the production and accumulation of oxidative stress in cells [18].

The limitation of the present study is ignoring the amount of glucose absorption in the cytoplasm after adding NAC. Previous studies show the effects of reduced bone differentiation and mineralization due to ROS generated by high glucose. High glucose damages the mitochondrial membrane potential and disrupts its function [19].

NAC is naturally found in small amounts in some fruits and vegetables. NAC benefits include increasing glutathione S-transferase activity, eliminating free radicals, stabilizing protein structures with cysteine disulfide bonds, and antioxidant and anti-inflammatory properties. NAC traps the ROS accumulated in the cell and blocks the factors that prevent ATP synthesis and mitochondrial transcriptional activity [20].

Increasing glucose level activates the ROS-AKT-mTOR pathway in the cell and disrupts the physiological processes of the cell, such as proliferation and osteogenic differentiation. It is still unclear whether NAC directly disrupts the ROS-AKT-mTOR signaling pathway or exerts this role indirectly [21].

Conclusion

Although NAC improves ADSCs' proliferation and osteogenic differentiation by reducing the effects of oxidative stress caused by high glucose, its mechanism should be investigated and more studied. According to the results, the protective role of NAC can be used for therapeutic purposes and restorative medicine in people with diabetes and improve the resulting bone problems.

Declarations

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article. The authors declare that there is no conflict of interest.

Authors' contributions

Zeinab Piravar and Mina Ramezani designed this study. Ramtin Hamidian obtained and analyzed the data. Zeinab Piravar, Mina Ramezani, and Ramtin Hamidian proceeded to the quality control and the manuscript drafting. Zeinab Piravar revised the final version.

Conflict of interest

The authors declare that there is no conflict of interest in this article.

Consent for publications

All authors agree to have read the manuscript and authorize the publication of the final version of the manuscript.

Availability of data and material

Data are available on request from the authors.

Funding/Support

Not applicable.

Ethics approval and consent to participate

The ethical approval was certified by Islamic Azad University Tehran Central Branch (Ethic Committee Reference NO.: IR.IAU.PS.REC. 1398.323).

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