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High Glucose in Culture Media of Adipose Derived Mesenchymal Stem Cells – Gene Expression Alteration and Early Senescence

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ABSTRACT

Subcutaneous adipose tissue derived mesenchymal stem cells (ASCs) are an attractive cell population for cell therapy applications. Cell culture conditions vary among reported protocols and can affect the quality of stem cells. The aim of this study was to determine the effect of glucose concentration on adipose tissue derived mesenchymal stem cells' culture. ASCs were isolated from subcutaneous fat of patients undergoing abdominoplasty. Cells were cultured with glucose concentration of 4.5 grams/liter or 1 gram/liter. The phenotypic characterization of the stem cells was evaluated by immunostaining and FACS analysis. Proliferation was assessed by division rate study. Senescence ratio was evaluated by beta galactosidase assay at an advanced passage. Gene profiling for senescence was performed by real time PCR and western blot. Multipotency ability was evaluated by differentiation studies. Culturing adipose tissue derived mesenchymal stem cells with high glucose resulted in reduced proliferative capacity (DMEM high glucose 53 ± 7.5 h, DMEM low glucose 32 ± 4 h), and increased senescence ratio (DMEM high glucose $50\% \pm 3$, DMEM low glucose $23.4\% \pm 9$). Gene profiling revealed decreased Ki67 and PCNA expression and western blot analysis revealed decreased NOX-4 expression in stem cells cultured with high glucose. Moreover, ASCs cultured with high glucose exhibited poorer differentiation potential in comparison to cells cultured with low glucose. In conclusion, culturing ASCs with high glucose resulted in decreased division rate and proliferative capacity, early senescence, lower expression of NOX-4, and reduced multipotency.

Keywords

Adipose derived mesenchymal stem cells (ASCs), Glucose, Hyperglycemia, Multipotency, Senescence.

Abbreviation list:

ASCs: Adipose tissue derived mesenchymal stem cells; MSCs: Mesenchymal stem cells; NOX-4: NADPH Oxidase 4; PCNA: Proliferating cell nuclear antigen.

Introduction

Adipose derived mesenchymal stem cells (ASCs) are an attractive population of stem cells for regenerative cell therapy since they can be isolated by relatively simple procedures [1], are easily expanded *ex vivo* and have the ability to differentiate to several cell types [2].

There are many different protocols for culturing ASCs *in vitro*, with each variation in culture conditions and in culture medium may affect the cells' proliferative capacity, differentiation potential and senescence [2,3]. Senescence is characterized by end stage growth arrest, morphological and cell-size changes, gene alteration, lack of DNA replication, increased activity of beta-galactosidase (**β–galactosidase**) and loss of cell ability to differentiate [4]. When isolating stem cells for cell therapy purposes, it is crucial to culture

the cells in such conditions that will provide good viability, with low senescence rate and consequently high proliferative capacity and multipotency.

Standard culture media contain varying glucose concentrations, from 1 to 4.5 grams/liter. Variations in glucose level may affect cellular function [5]. Hyperglycemic environment leads to a cascade of cellular events that increases cells' vulnerability to oxidative stress, interferes with the function of intracellular proteins and influences transcription factors that subsequently alter gene expression [6].

It was shown that hyperglycemia suppress hematopoietic activity in bone marrow mesenchymal stem cells (MSCs) [7]. Moreover, high glucose in culture medium decreases MSCs' proliferation rate and differentiation potential and may cause genomic instability [8-10].

This study aims to analyze the effect of high glucose on ASCs' phenotypic characterization, division rate, senescence, multipotency and gene profiling.

Materials and Methods
Donors and isolation of tissue
Adipose tissue was harvested from the subcutaneous fat of six women who underwent abdominoplasty. The mean age of the patients was 46.6 years (SE, 7 years; range, 37–57 years). Mean body mass index of donors was 28.8 kg/m^2 (SE, 3.9 kg/m^2; range 24.5–34.3 kg/m^2). Adipose tissue from each patient was subjected to power assisted liposuction (PAL). Briefly, a small incision was made and a blunt hollow cannula, 3 mm in diameter and 30 cm long, was introduced to the subcutaneous space (PAL-200E MicoAire power-assisted lipoplasty device, MicroAire Surgical Instruments LLC, Charlottesville, VA, USA). Gentle suction was performed by applying negative pressure of less than 1 Atm, in combination with tumescent technique; 1mg of Adrenalin + 400 mg of Lidocaine in 1 liter of saline. Adipose tissue was collected in a sterile container.

Cell isolation and culture
ASCs were isolated as previously described [1]. Briefly, 15 g of tissue or lipoaspirate were washed with phosphate buffered saline (PBS). The tissue was minced with surgical scissors to pieces of approximately 1 cm^2. Cells were extracted by collagenase digestion (0.075% collagenase type I, Sigma-Aldrich, St. Louis, MO, USA) for 45 minutes at 37°C with gentle shaking. Collagenase was then neutralized with Dulbecco's Modified Eagle's medium (DMEM, Biological industries, Beit Haemek, Israel) containing 10% fetal bovine serum (FBS). Digested fat was centrifuged, and the cell pellet was resuspended in basal medium for adipose-derived stem cells (Lonza, Walkersville, MD, USA).

Nucleated cells were stained with a solution of 3% acetic acid and methylene blue (Stemcell Technologies, Vancouver, Canada) and were counted manually by means of a high-power light microscope. Isolated cells were seeded into 10-cm tissue culture plates (BD Falcon, BD Biosciences, San Jose, CA, USA) at a density of 1x10^5 cells per cm^2. Cells were cultured with either DMEM high glucose (4.5 grams/liter, Biological industries) or DMEM low glucose (1 grams/liter, Biological industries). Non adherent cells were removed 36 hours after plating. The cell culture medium was changed every 3 days. When cultures reached 100% confluence, cells were detached with trypsin-EDTA (Sigma-Aldrich), and cultures were split 1:2.

ASCs characterization by Fluorescence-Activated Cell Sorting (FACS) Analysis
Cultured ASCs at passages zero and three that were 100% confluent were detached with trypsin, equally dispensed into FACS tubes (1x10^5 cells per tube), and incubated with monoclonal antibodies against human CD45 (Dako, Carpinteria, CA, USA), CD90 (Dako), CD73 (BD Pharmingen, BD Biosciences), CD105 (eBioscience, San Diego, CA, USA) or with isotype control antibodies. All antibodies were conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Cells were then analyzed by flow cytometry for the expression of cell surface markers.

ASCs division rate
ASCs at passage three were seeded at a density of 1x10^4 cells per well in six well plates (Falcon, Corning, NY, USA). When the cells reached 70-80% confluence, the logarithmic growing phase of the cells was determined using the following formula: Td = (t2 - t1) x log(2)/log(q2/q1). Whereas (t2 –t1) equals the time of incubation, (q1) is the initial number of cells and (q2) is the final amount of the cells.

ASCs proliferative capacity
Total RNA was isolated from ASCs at passage six, using High Pure RNA Isolation kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. cDNA was synthesized using Verso cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Real-time quantitative RT–PCR Syber-Green assays were performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). All reactions were done in triplicates with the use of the fast Syber-Green Master Mix (Applied Biosystems) and primers for the mRNA of PCNA and Ki67 (Sigma-Aldrich). The primer sequences were as follows: PCNA: Fwd 5'-GAAG-CACCAAACCAGGAGAA-3'; Rev 5'-CCGTCTTTTGCACAG-GAAAT-3'; Ki67: Fwd 5'-GCTTCTCTTCTGACCCTGATGA-3'; Rev 5'-TTCCCTGAGCAACACTGTCT-3'). All data were analyzed with StepOne Software v2.3.

Assessment of Multipotency
ASCs at passage three were assessed for their capacity to differentiate into osteocytes or adipocytes. Cells were seeded into a 24-well plate at a density of 1x10^4 cells per well, and differentiation medium was added when the cells reached 100% confluence. For adipocyte differentiation, cells were incubated with 10% FBS, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 10 μg/mL insulin, and 100 μM indomethacin in DMEM high glucose. For osteocyte differentiation, StemPro Osteogenesis Differentiation basal medium (Gibco, Thermo Fisher scientific) was added.

The medium was changed twice a week for two or three weeks to induce ASCs to differentiate into osteocytes or adipocytes, respectively. Differentiation into adipocytes was assessed with Oil Red O stain (Sigma-Aldrich), an indicator of intracellular lipid accumulation. Cells were fixed at room temperature for 20 minutes in 4% paraformaldehyde and then were incubated in 0.5% (wt/vol) Oil Red O in 100% isopropanol at room temperature for 10 minutes. Excess stain was removed by several washes with distilled water. Differentiation into osteocytes was assessed with 2% Alizarin Red (Sigma-Aldrich), which stains calcium deposits. The pH of Alizarin Red was adjusted to approximately 4.2 with 10% hydrochloric acid and 10% ammonium hydroxide. Cells were fixed with 4% paraformaldehyde for 20 minutes and then were stained with Alizararin Red for 15 minutes at room temperature. Excess stain was removed by several washes with distilled water. Images of stained cells with either Oil Red O or Alizarin Red were taken with a light microscope and quantified as the percentage of stained area per high-power field with ImageJ software.

Senescence by β-galactosidase Activity

Cultured ASCs (1×10^5 cells) at passage four were seeded into a six-well plate. When cells reached 70-80% confluence, the medium was replaced and β-galactosidase activity was assessed as previously described [10] with a Senescence Detection kit (Biovision, Milpitas, CA, USA) according to the manufacturer's instructions. Briefly, cells were placed in a fixative solution for 15 minutes and then were incubated with β-galactosidase stain at 37°C for 12 hours. Images of senescent cells, identified by blue staining, were taken with a light microscope (Nikon eclipse 50i, Nikon Instruments, Tokyo, Japan). Cells were counted in five different fields for each group. Researchers were blinded to the groups. Senescent cells were counted from duplicate samples obtained from three donors, and the experiments were repeated three times.

Western blot for NOX-4

ASCs at passage four were harvested and their proteins extracted by RIPA buffer (Sigma-Aldrich) and sonication. Western blot analysis was performed using the Mini-PROTEAN® Electrophoresis System and Mini Trans-Blot® Cell system (Bio-Rad Laboratories, Hercules, CA, USA). 10% polyacrylamide gels were prepared and each lane was loaded with an equal amount of protein extracts (40μg), which, following electrophoresis, were transferred to an Immobilon PVDF transfer membrane (Millipore, Billerica, MA, USA). Membranes were then probed with anti-NADPH oxidase 4 (NOX-4) antibody (1:5000; Abcam, Cambridge, England), overnight, and then incubated with goat anti rabbit HRP-conjugated secondary antibody (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour. The intensity of the signal was determined using the EZ-ECL detection system (Biological Industries).

Statistical Analysis

Comparison between groups was performed by student's t-test. Level of significance was set at $p < 0.05$ (*). Results are expressed as mean ± SD.

Results

Characterization by surface phenotype

ASCs were cultured with DMEM high glucose and DMEM low glucose. Cells were immunostained for the cell surface markers CD45, CD73, CD90, and CD105 at passage zero and at passage three. No significant difference was observed between cells grown in high and low glucose (Figure 1).
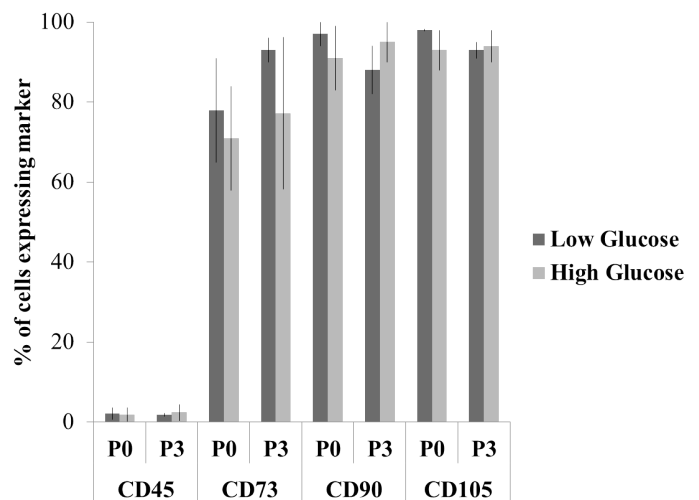
Figure 1. Immunophenotyping and FACS analysis for mesenchymal stem cells markers.
Cultured ASCs at passage 0 and 3 were detached with trypsin, equally dispensed into FACS tubes (1×10^5 cells per tube), and incubated with monoclonal antibodies against human CD45, CD73, CD90 and CD105. Cells were then analyzed by flow cytometry for the expression of cell surface markers. CD: cluster of differentiation.
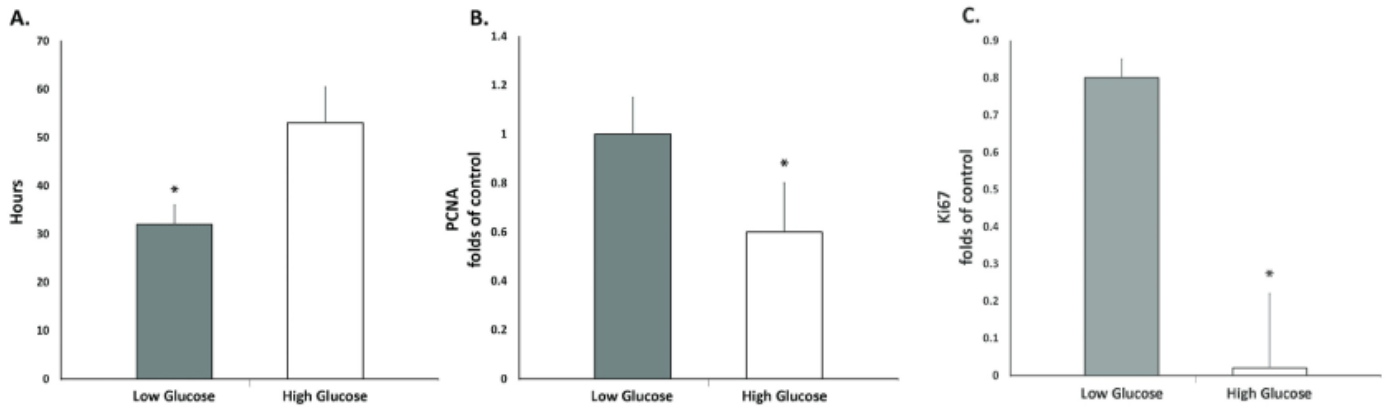
Division rate

According to the logarithmic phase of the dividing cells, ASCs cultured with DMEM high glucose exhibited a decreased division rate in comparison to cells cultured with low glucose. (DMEM high glucose 53 ± 7.5 hours, DMEM low glucose 32 ± 4 hours, p=0.002) (Figure 2A).

PCNA and Ki67 gene expression levels

The effect of different culture media on the proliferative capacity of ASCs was examined by the expression levels of the hallmark proliferation markers PCNA and Ki67. Cells that were cultured in DMEM high glucose exhibited minimal levels of Ki67 and significantly lower levels of PCNA in comparison to cells cultured with low glucose. (PCNA: DMEM low glucose 1 ± 0.15, DMEM high glucose 0.6 ± 0.2, p<0.05. Ki67: DMEM low glucose 0.8 ± 0.05, DMEM high glucose 0.02 ± 0.2, p<0.05) (Figure 2B and 2C).
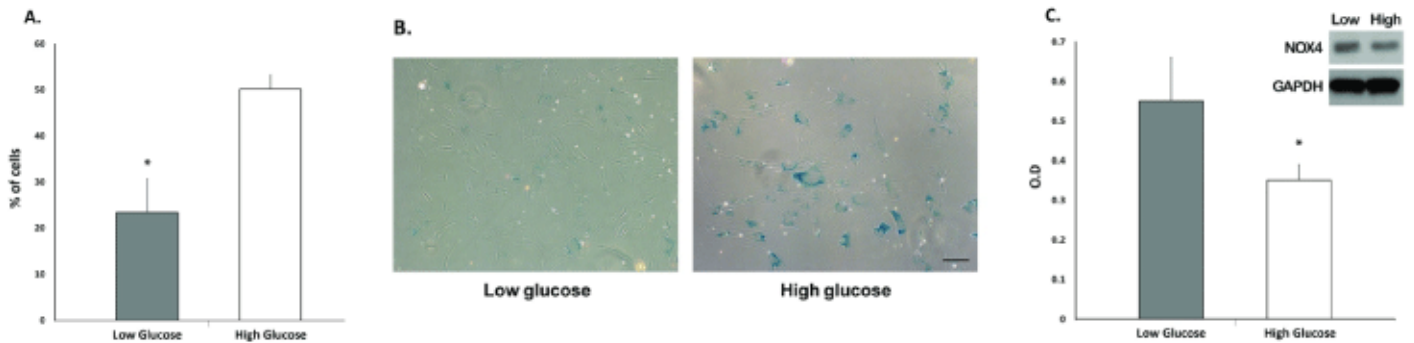
Senescence by β- galactosidase

The senescence ratio of ASCs cultured in the different media was examined. We demonstrated a significant increase in senescence rate of ASCs when cultured with DMEM high glucose evident by β-galactosidase activity. (DMEM low glucose: 23.4% ± 7.3 of cells, DMEM high glucose: 50.2% ± 3 of cells, p=0.001) (Figure 3A and 3B).

Figure 2. Division rate and gene expression.
A. ASCs at passage 3 were seeded at a density of 1×10^4 cells per well in a six-well plate. Division rate was assessed by calculating the logarithmic growing phase of the cells. B, C. qRT-PCR for ki67 and PCNA levels in ASCs at passage 3 (*p<0.05).

Figure 3. Senescence and NOX-4 expression levels.
A. ASCs at passage 4 were seeded in 6 wells plates. β-Galactosidase activity was detected by staining with β-Galactosidase reaction buffer. Senescent cells (dyed blue) were quantified by light microscopy. Results are presented as percentage of total dyed cells in high power field. B. Representative images of β- Galactosidase staining of ASCs (Bar=200μm). C. Western blot analysis of NOX-4 levels in ASCs at passage 4 (*p<0.05).

Evaluation of NOX-4 expression
NOX-4 was previously shown to play a key role in sensing glucose levels. Low levels of glucose in the media resulted in NOX-4 increased activity [11], and increased NOX-4 activity results in increased Erk and p38 activity [12]. Indeed, western blot analysis revealed that NOX-4 protein levels were significantly higher in ASCs cultured with low glucose in comparison to cells cultured in high glucose (Figure 3C). Quantitative analysis of the results showed a 1.55-fold increase of NOX-4 in ASCs cultured in low glucose (p<0.05).
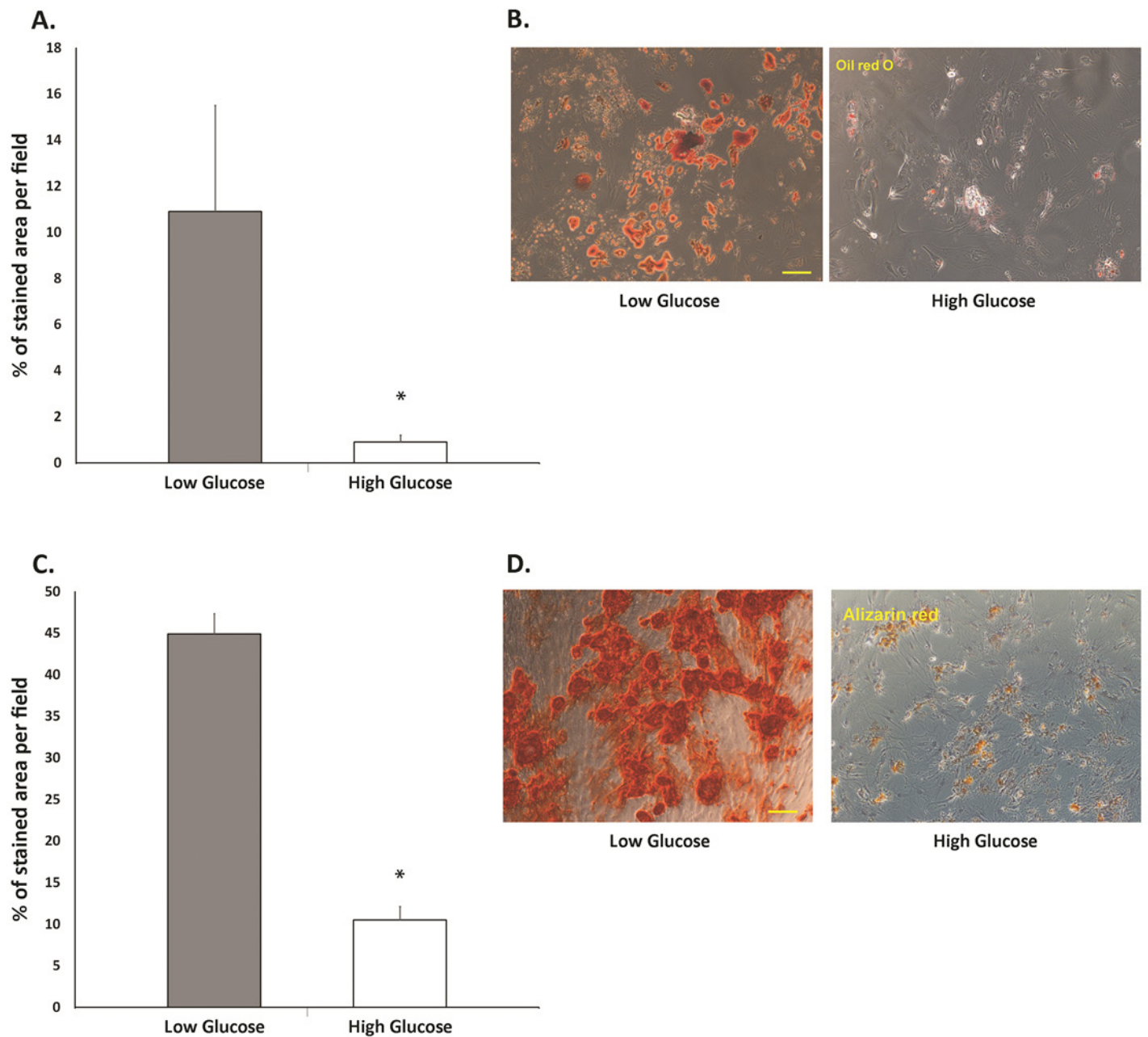
Multipotency
ASCs were cultured with DMEM either with low glucose or high glucose until confluence. Then the cells were cultured in bone or adipose differentiation medium for two or three weeks, respectively. Differentiation to adipocytes or osteocytes was quantified using Oil Red O staining and Alizarin red staining, respectively. Cells that were cultured with DMEM high glucose exhibited lower capacity to differentiate into adipocytes and osteocytes as compared to cells cultured with DMEM low glucose (Adipose: DMEM low glucose: 10.9% ± 4.6 of stained area, DMEM high glucose: 0.9% ± 0.3 of stained area, p=0.0001. Bone: DMEM low glucose: 45% ± 2.4 of stained area, DMEM high glucose: 10.5% ± 1.6 of stained area, p=0.007) (Figure 4).

Discussion
In this study we compared the effect of growing ASCs in high glucose media and low glucose media. We demonstrated that ASCs grown in high glucose media resulted in decreased proliferation rate and multipotency ability, compared to ASCs grown in low glucose. Accordingly, senescence rate was higher in ASCs cultured with high glucose, evident by increased β-galactosidase activity. Gene profiling for ASCs cultured in high glucose demonstrated decreased Ki67, PCNA and NOX-4 expression compared to ASCs cultured in low glucose.

Phenotypic characterization of ASCs revealed similarity in CD45, CD73, CD90 and CD105 expression between ASCs cultured with high glucose or low glucose, at passage zero and at passage three. These findings are in line with previous studies reporting similar expression patterns of surface markers in mesenchymal stem cells derived from either adipose tissue or bone marrow, cultured with different kinds of cell culture media [13-15].

A.

B. Low Glucose / High Glucose (Oil red O)

C.

D. Low Glucose / High Glucose (Alizarin red)

Figure 4: Multipotency of ASCs cultured with either high or low glucose.
A. ASCs at passage 3 were subjected to adipocytes differentiation and stained with Oil red O. Images were obtained by a light microscope and quantified with ImageJ software. Results are presented as the percent of stained area from entire area of a high power field (* $p<0.05$). B. Representative images of Oil red O staining (Bar=200μm). C. ASCs at passage 3 were subjected to osteocytes differentiation and stained with Alizarin red. Images were obtained by a light microscope and quantified with ImageJ software. Results are presented as the percent of stained area from entire area of a high power field (*$p<0.05$). D. Representative images of Alizarin red staining (Bar=200μm).

Glucose levels are known to influence regulation of genes, and may affect cell apoptosis and differentiation. Glucose levels were shown to interfere with epidermal growth factor and subsequently influence proliferation rates [16]. Stozling et al. demonstrated that low glucose levels resulted in enhanced proliferative capacity [9]. Moreover, it is known that senescence may be induced by high glucose levels [17]. In this study we showed that high glucose levels not only prolonged ASCs' division rate, but also caused a decrease in the expression of the markers of proliferation Ki67

and PCNA [18]. Adding to that, our results demonstrate higher senescence ratio in ASCs cultured with high glucose, evident by increased beta galactosidase activity.

It was previously shown that NOX-4 activity mediates cell proliferation, and that NOX dependent ROS generation activates growth factors that lead to an increase in cell proliferation and survival [19,20]. Moreover, NOX activity is linked to cell survival via inactivation of phosphatases by NOX derived ROS

and upregulation of survival pathways such as Janus kinase [21], or through activation of insulin-like growth factor 1 (IGF1) and subsequently increase of anti-apoptotic pathways [22]. Interestingly, Owada and colleagues previously reported a direct increase in activity of NOX-4 in glucose deprivation state [11]. Taken together, these data are in line with our results in the present study, demonstrating increased proliferative capacity and decreased senescence ratio which correlated with increased NOX-4 expression in ASCs cultured with low glucose compared to ASCs cultured in high glucose.

MSCs are multipotent, but that quality diminishes over time in culture. Loss of multipotency was associated with advance glycation end products [23], and low glucose conditions were shown to contribute to improved differentiation potential of MSCs [24]. We also demonstrated a significant reduction in multipotency in ASCs cultured with high glucose that confirmed previous results that demonstrated that high glucose incubated- nucleus pulpous-derived mesenchymal stem cells (NPMSC) showed significantly decreased cell proliferation, colony formation ability, migration and wound-healing capability compared with those of low glucose incubated-NPMSC whereas increased cell apoptosis, cell senescence [25].

To date, there is no consensus on one protocol for *in vitro* culture of ASCs, and result discrepancies from different laboratories may be related to variations in culture conditions. We conclude that high glucose in culture medium (4.5 grams/liter) provide sub optimal conditions for culturing ASCs, as ASCs express low levels of genes involved in proliferation and cell survival like Ki67, PCNA and NOX-4. Accordingly, ASCs cultured in high glucose exhibited poor proliferation, early senescence, and low ability to differentiate.

Thus, these findings suggest a potential advantage for culturing ASCs with low glucose levels.

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