Insulin-like growth factor-1 promotes the proliferation and odontoblastic differentiation of human dental pulp cells under high glucose conditions

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Abstract. Insulin-like growth factor-1 (IGF-1) promotes human dental pulp stem cell proliferation and osteogenic differentiation. However, the effects of IGF-1 on the proliferation, apoptosis and odontoblastic differentiation (mineralization) of dental pulp cells (DPCs) under high glucose (GLU) conditions remain unclear. In this study, isolated primary human DPCs were treated with various concentrations of high GLU. Cell proliferation and apoptosis were determined by Cell Counting Kit-8 and Annexin V-FITC/PI assays, respectively. The cells were cultured in odontoblastic induction medium containing various concentrations of high GLU. Odontoblastic differentiation was determined by alkaline phosphatase (ALP) activity assay. Mineralization formation was evaluated by von Kossa staining. The expression levels of IGF family members were measured by western blot analysis and RT-qPCR during proliferation and differentiation. The cells were then exposed to 25 mM GLU and various concentrations of IGF-1. Cell proliferation, apoptosis, ALP activity, mineralization formation and the levels of mineralization-related proteins were then evaluated. Our results revealed that high GLU significantly inhibited cell proliferation and promoted cell apoptosis. GLU (25 and 50 mM) markedly reduced ALP activity and mineralization on days 7 and 14 after differentiation. The levels of IGF family members were markedly decreased by high GLU during proliferation and differentiation. However, IGF-1 significantly reversed the effects of high GLU on cell proliferation and apoptosis. Additionally, IGF-1 markedly restored the reduction of ALP activity and mineralization induced by high GLU. Our findings thus indicate that IGF-1 attenuates the high GLU-induced inhibition of DPC proliferation, differentiation and mineralization.

Introduction

Dental pulp participates in the reparative regeneration of the tooth tissues (1). Dental pulp tissue contains multiple cells that possess plasticity and multipotency, including fibroblasts, inflammatory and immune cells, odontoblasts and undifferentiated mesenchymal cells (1-3). Fibroblasts and odontoblasts are the main cell types in dental pulp (4). In response to stimuli or injuries, dental pulp cells (DPCs) differentiate into odontoblasts to replace the necrotic cells and then generate reparative dentin (5).

Diabetes mellitus (DM) is a severe chronic disease that markedly affects the health and life quality of individuals (6). DM is a multi-organ and multi-factorial metabolic disease that is characterized by absolute or relative deficiency in insulin secretion, insulin resistance and β -cell dysfunction, which may ultimately lead to hyperglycemia (7-9). DM is a major cause of morbidity and mortality worldwide (10). Approximately 87 million adults suffered from the disease in 2014. The number of individuals with DM is expected to increase to almost 592 million by the year 2035 (11). DM may lead to numerous complications, including cardiovascular diseases, microvascular diseases (such as diabetic retinopathy), osteoporosis and diabetic nephropathy (12,13). Additionally, DM also affects the functions of dental pulp and periapical tissues (14,15). A previous study indicated that DM can result in the necrosis of dental pulp and in the development of periapical lesions in diabetic rats (16). Long-term DM has been demonstrated to increase the basement membranes thickness of dental pulp vessels and to contribute to the occurrence and development of angiopathy (17). It also been demonstrated that rats with streptozotocin (STZ)-induced diabetes exhibit a significant reduction in pulpal blood flow (18). Moreover, hyperglycemia inhibits dentin bridge formation and enhances inflammatory cell infiltration in diabetic rats (19).

Insulin-like growth factor-1 (IGF-1), a member of the insulin-like peptide family, plays a vital role in the survival, apoptosis and differentiation of cells within various organs, including teeth (20-22). Joseph *et al* found that secretory ameloblasts, secretory odontoblasts and mature ameloblasts express high levels of IGF-1 in the development of the rat incisor (23). It has also been demonstrated that IGF-1 promotes human dental pulp stem cell proliferation and osteogenic differentiation by

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increasing the expression of differentiation markers through the mammalian target of rapamycin (mTOR) signaling pathway (24). IGF-1 has also been reported to protect DPCs against the cytotoxicity of composite materials via endogenous antioxidant mechanisms (25).

In the present study, we cultured primary human DPCs in order to investigate the effects of high glucose (GLU) on the proliferation, apoptosis and differentiation/mineralization of DPCs. We further evaluated the protective effects of IGF-1 under high GLU conditions. Our study provides insight into possible treatment options to counteract the oral complications associated with DM.

Materials and methods

Isolation of human DPCs. Human premolars extracted for orthodontic purposes and third molars from patients without dental carious and periodontal problems (a total of 80 adults; age: 18-25 years old) were obtained from China Medical University School and Hospital of Stomatology. Dental pulp tissues were obtained from teeth and washed with phosphate-buffered saline (PBS). All study protocols were approved by the Ethics Committee of China Medical University. Written informed consent was written from all patients. The tissues were then cut into sections and digested with 0.3% type I collagenase and 0.4% dispase at 37°C for 1 h. The isolated human DPCs were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Inc., Logan, UT, USA) at 37°C in a 5% CO₂ atmosphere.

Odontoblastic differentiation. The cells at passage 3 were seeded on culture dishes. After being grown to 70-80% confluence, the cells were cultured in odontoblastic induction medium (OM) for 1, 3, 7 and 14 days, composed of DMEM, 50 µg/ml ascorbic acid and 10 mM β-glycerophosphate sodium (both from Sigma-Aldrich, St. Louis, MO, USA).

Cell proliferation assay. The DPCs were seeded into 96-well plates at a density of 2x10³ cells/well and cultured in DMEM containing various concentrations of GLU (10, 25 and 50 mM) (Sigma-Aldrich). The cells were cultured for 24, 48 and 72 h. Cell Counting Kit-8 (CCK-8) agent (10 µl) (Beyotime Institute of Biotechnology, Inc., Haimen, China) was then added to each well and the cells were incubated for 1 h at 37°C. The absorbance was measured at 450 nm using a microplate reader (ELX-800; BioTek Instruments, Inc., Winooski, VT, USA). The optimum concentration of GLU was determined.

In addition, the cells were plated in 96-well plates (2x10³ cells/well) and maintained in DMEM containing high GLU (25 mM). The dose of GLU (25 mM) used was the minimum effective dose. The cells were then treated with various concentrations of IGF-1 (10, 50 and 100 ng/ml) for 24, 48 and 72 h (Peprotech Inc., Rocky Hill, NJ, USA). Cell proliferation was evaluated by CCK-8 assay (Beyotime Institute of Biotechnology, Inc.) according to the manufacturer's instructions.

Cell apoptosis assay. The cells were harvested after the corresponding treatments and washed twice with PBS.

Following centrifugation at 800 rpm for 5 min, the cells were resuspended in 500 µl binding buffer gently. Subsequently, 5 µl Annexin V-FITC and 5 µl propidium iodide (PI) (KeyGen Biotech Co., Nanjing, China) were added to the suspension and mixed immediately. The cell suspension was incubated at room temperature for 15 min in the dark and analyzed by flow cytometry (Model C6; BD Biosciences, San Jose, CA, USA).

Alkaline phosphatase (ALP) activity assay. The cells were resuspended in 100 µl PBS and subjected to repeated freeze-thawing cycles. The supernatant was obtained by centrifugation at 12,000 rpm for 10 min and quantified by BCA (Wanleibio, Shenyang, China). The activity of ALP was measured using an ALP assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions and expressed as U/g protein.

von Kossa staining. The fixed cells were washed 3 times with deionized water. Subsequently, the cells were stained with 1% silver nitrate (Jizhun, Shanghai, China) and exposed to ultraviolet light for 20 min. After washing with deionized water, the cells were incubated for 5 min with 5% sodium thiosulfate and counterstained for 30 sec with 0.1% nuclear fast red (both from Sinopharm Chemical Reagent Co., Ltd., Beijing, China). The coverslips were dehydrated in ethanol (75, 85, 95 and 100%) and captured under a microscope (DP73; Olympus, Tokyo, Japan).

Western blot analysis. Total proteins were extracted from the cells using the total protein extraction kit purchased from Wanleibio and quantified. Total proteins (40 µg in each lane) were subjected to 8, 10 or 15% SDS-PAGE (Wanleibio), followed by transfer onto PVDF membranes (EMD Millipore, Bedford, MA, USA). The membranes were blocked with non-fat milk and then incubated at 4°C overnight with antibodies against osteocalcin (OCN) (1:200; sc-376835), osteonectin (ON) (1:200; sc-398419), dentin matrix protein-1 (DMP-1) (1:200; sc-73633) (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), osteopontin (OPN) (1:500; D121078; Sangon Biotech Co., Ltd., Shanghai, China), dentin sialoprotein (DSP) (1:500; bs-8557R; BIOSS, Beijing, China), IGF-1 (1:400; BA0939), IGF-1 receptor (IGF-1R) (1:400; BA0498), IGF-binding protein 1 (IGFBP1) (1:400; BA1749) and IGFBP3 (1:400; BA3641) (all from Boster Biological Technology, Ltd., Wuhan, China). The membranes were washed and incubated at 37°C for 45 min with goat anti-rabbit/mouse horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000; WLA023/WLA024; Wanleibio). The protein bands were visualized by using enhanced chemiluminescence (ECL) substrate (Wanleibio) and quantified using Gel-Pro Analyzer 4.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using the RNApure total RNA extraction kit (BioTeke Corp., Beijing, China) and 1 µg total RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (BioTeke Corp.) according to the manufacturer's instructions. The primers used in this study were synthesized by Sangon Biotech Co., Ltd. and were as follows: IGF-1 forward, 5'-ACAAGCCCCACAGGGTATG-3'

Figure 1. Effect of high glucose (GLU) on the proliferation and apoptosis of human dental pulp cells (DPCs). (A) The proliferative ability of DPCs was determined by Cell Counting Kit-8 (CCK-8) assay at 24, 48 and 72 h following exposure to high GLU (10, 25 and 50 mM). (B) Cell apoptosis was measured by Annexin V-FITC/propidium iodide (PI) staining assay. *P<0.05 and **P<0.01 vs. control group.

and IGF-1 reverse, 5'-CACTCCCTCTACTTGCGTTCT-3'; IGF-1R forward, 5'-TGCTGTATGCCTCTGTGAACC-3' and IGF-1R reverse, 5'-AGACCATCCCAAACGACCC-3'; IGFBP1 forward, 5'-CCTGCCAAACTGCAACAAG-3' and IGFBP1 reverse, 5'-CCCATTCCAAGGGTAGACG-3'; IGFBP3 forward, 5'-TAAGGTGGAGTCCTACTTGT-3' and IGFBP3 reverse, 5'-ACTTGTGATGCCTCTGAATG-3'; and β -actin forward, 5'-CTTAGTTGCGTTACACCCTTTC TTG-3' and β -actin reverse, 5'-CTGTCACCTTCACCGT TCCAGTTT-3'. qPCR amplification (95°C for 10 min, followed by 40 cycles of 95°C for 10 sec, 60°C for 20 sec and 72°C for 30 sec) was performed on an Exicycler™ 96 quantitative fluorescence analyzer (Bioneer Co., Daejeon, Korea) using SYBR-Green (Solarbio, Beijing, China). Gene expression levels were normalized to β -actin levels and calculated using $2^{-\Delta\Delta C_t}$ method (26).

Statistical analysis. Data are expressed as the mean $\pm$ SD. All data were analyzed by one-way analysis of variance (ANOVA) and Bonferroni's multiple comparison test. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

High GLU inhibits the proliferation and promotes the apoptosis of human DPCs. In this study, CCK-8 assay was performed to evaluate the effects of high GLU on the proliferation of DPCs. As shown in Fig. 1A, exposure to high GLU (at concentrations of 25 and 50 mM) for 24, 48 and 72 h significantly decreased the viability of the DPCs compared with the control group. The effects of high GLU on cell apoptosis were also investigated by Annexin V-FITC/PI staining. As shown in Fig. 1B, exposure to GLU (10, 25 and 50 mM) markedly promoted the apoptosis of the DPCs compared with the control group, as evidenced by the increased apoptotic rate.

High GLU inhibits the odontoblastic differentiation of and mineralization in DPCs. No statistically significant differ-

ences were observed in the activity of ALP among the groups on days 1 and 3 (Fig. 2A). ALP activity in the cells cultured in OM was significantly higher than that of the untreated control cells on days 7 and 14. Exposure to high GLU (25 and 50 mM) significantly reduced this increase from day 7 after differentiation. Mineralization in DPCs was assessed by von Kossa staining. Our data indicated that mineralized matrix formation in the cells cultured in OM was markedly enhanced compared with the control group on days 7 and 14 (Fig. 2B). However, exposure to high GLU (25 and 50 mM) significantly inhibited odontoblastic mineralization.

High GLU reduces the expression levels of IGF family members in DPCs. A previous study reported that IGF-1 can promote the proliferation and osteogenic differentiation of human dental pulp stem cells (24). Therefore, we examined the expression levels of several IGF family members in the DPCs after the indicated treatments. The cells were cultured in normal DMEM and exposed to increasing concentrations of GLU for 24 h. Compared with the control group, there was a significant reduction in the IGF-1, IGF-1R, IGFBP1 and IGFBP3 protein levels in the GLU-exposed cells (Fig. 3A). Consistent with the results of western blot analysis, the corresponding decreases were confirmed by RT-qPCR (Fig. 3B).

Furthermore, the cells were maintained in differentiation medium. After 7 days of differentiation, the mRNA and protein levels of IGF-1, IGF-1R, IGFBP1 and IGFBP3 in the OM + GLU groups were markedly decreased in comparison with the OM group, as evaluated by western blot analysis (Fig. 3C) and RT-qPCR (Fig. 3D). Several days after differentiation, the protein levels of IGF-1 (days 7 and 14), IGF-1R (days 3, 7 and 14), IGFBP1 (days 7 and 14) and IGFBP3 (days 7 and 14) in the OM + 25 mM GLU group were significantly lower than those in the OM group (Fig. 3E). Markedly decreased mRNA levels were firstly observed on day 3 (Fig. 3F).

IGF-1 abolishes the effect of high GLU on the proliferation and apoptosis of DPCs. The cells were then exposed to 25 mM

Figure 2. Effect of high glucose (GLU) on the differentiation and mineralization of dental pulp cells (DPCs). (A) The cells were cultured with odontoblastic induction medium (OM) containing high GLU. Alkaline phosphatase (ALP) activity was measured on days 1, 3, 7 and 14 after differentiation. (B) The mineralized nodules in the cells were evaluated by von Kossa staining on days 3, 7 and 14 after differentiation induction. Scale bar, 200 μ m. ** P <0.01 vs. control group; # P <0.05 and ## P <0.01 vs. OM group.

GLU and various concentrations of IGF-1. As shown in Fig. 4A, in the presence of IGF-1 at concentrations of 50 and 100 ng/ml, cell viability was significantly increased compared with the 25 mM GLU group. As expected, the pro-apoptotic effects of high GLU were markedly suppressed by treatment with 100 ng/ml IGF-1 (Fig. 4B).

IGF-1 attenuates the effect of high GLU on the odontoblastic differentiation of and mineralization in DPCs. Subsequently, the cells were cultured for different periods of time in OM with 25 mM GLU and IGF-1. The results demonstrated that the inhibitory effects of high GLU on ALP activity (Fig. 5A) were markedly abolished by IGF-1 (50 and 100 ng/ml) on day 7 after odontoblastic induction. Treatment with various concentrations of IGF-1 reversed the effects of high GLU on mineralization in DPCs. However, the difference was not statistically significant (Fig. 5B).

IGF-1 restores the decreased levels of mineralization-related proteins induced by high GLU. Furthermore, western blot analysis was used to measure the expression levels of mineralization-related proteins. Our results revealed that the OCN, ON, OPN, DSP and DMP-1 levels in the OM group were significantly higher than those in the control group (Fig. 6). In

the presence of 25 mM GLU, the levels of these mineralization-related proteins in the OM + 25 mM GLU group were markedly decreased compared with the OM group. However, IGF-1 treatment significantly restored the high GLU-induced decrease in the levels of mineralization-related proteins, including OCN, ON, OPN, DSP and DMP-1.

Discussion

The process of dentinal regeneration involves the proliferation and differentiation of DPCs into odontoblasts, dental pulp healing and reparative dentin formation (27). A high sucrose diet affects the dentin-pulp complex and reduces dentin formation, which contributes to caries in dentin (28). The effect of high GLU on the proliferation, apoptosis and differentiation of DPCs warrants further investigation. In the present study, we demonstrated that high GLU suppressed the proliferation, induced the apoptosis and inhibited the differentiation of human DPCs, accompanied by reduced levels of IGF-1 family members, including IGF-1, IGF-1R, IGFBP1 and IGFBP3. We then evaluated the effects of IGF-1 on the biological properties of DPCs under high GLU conditions. We found that IGF-1 treatment reversed the effects of high GLU on DPCs.

Figure 3. Effect of high glucose (GLU) on the expression levels of insulin-like growth factor-1 (IGF-1) family members. (A) Dental pulp cells (DPCs) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing various concentrations of GLU (10, 25 and 50 mM) for 24 h. Total proteins were extracted from the cells and the expression levels of IGF-1, IGF-1 receptor (IGF-1R), IGF-binding protein 1 (IGFBP1) and IGFBP3 were then measured by western blot analysis. β-actin was used as a loading control. (B) The mRNA levels of IGF-1 family members were measured by RT-qPCR and normalized against the β-actin level. (C) DPCs were cultured in odontoblastic induction medium (OM) containing various concentrations of GLU (10, 25 and 50 mM) for 7 days. The protein levels of IGF-1 family members were analyzed by western blot analysis. (D) RT-PCR analysis of IGF-1, IGF-1R, IGFBP1 and IGFBP3 mRNA levels. (E) Western blot analysis of the levels of IGF-1 family members on days 1, 3, 7 and 14 after differentiation. (F) RT-qPCR analysis of the levels of IGF-1 family members. * $P < 0.05$ and ** $P < 0.01$ vs. control group; # $P < 0.05$ and ## $P < 0.01$ vs. OM group.

It has been reported that hyperglycemia inhibits pulp repair (29). A previous study demonstrated that a high concentration of D-GLU (30 mM) markedly inhibited the proliferation of MD10-F2 pulp cells compared with the controls *in vitro* (30). Furthermore, previous studies have also demonstrated that high GLU (25 mM) reduces the proliferative capability of human cavernous endothelial cells (HCECs) and induces cell apoptosis *in vitro* (31,32). Our experiments consistently demonstrated that high GLU, particularly at the concentrations of 25 and 50 mM, markedly suppressed human DPC proliferation and promoted cell

apoptosis. ALP is a marker of odontoblastic differentiation and its activity is enhanced during odontoblastic induction (33,34). We found that high GLU inhibited ALP activity and mineralization in DPCs, suggesting that high GLU inhibited the proliferation and differentiation and induced the apoptosis of DPCs.

IGF-1 is an ubiquitous peptide hormone and an important anti-apoptotic factor (35). IGFBPs constitute multiple proteins that bind to IGFs and modulate the interaction of IGFs with their receptors (36). Yu *et al* reported that high GLU leads to cardiomyocyte H9c2 cell apoptosis by decreasing IGF-1R

Figure 4. Effect of insulin-like growth factor-1 (IGF-1) on the proliferation and apoptosis of human dental pulp cells (DPCs) under high glucose (GLU) conditions. (A) The cells were incubated with 25 mM GLU and IGF-1 (10, 50 and 100 ng/ml). Cell proliferation was evaluated by Cell Counting Kit-8 (CCK-8) assay. (B) Apoptosis was analysis by Annexin V-FITC/propidium iodide (PI) staining assay. * $P < 0.01$ vs. 25 mM GLU group.

Figure 5. Effect of insulin-like growth factor-1 (IGF-1) on the differentiation and mineralization of dental pulp cells (DPCs). (A) The cells cultured with odontoblastic induction medium (OM) were incubated with IGF-1 under high glucose (GLU) conditions. Alkaline phosphatase (ALP) activity was determined on days 1, 3, 7 and 14 after odontoblastic differentiation induction. (B) von Kossa staining of DPCs treated with high GLU (25 mM GLU) and IGF-1 in OM. Scale bar, 200 μ m. # $P < 0.05$ and ## $P < 0.01$ vs. OM group; && $P < 0.01$ vs. OM+25 mM GLU group.

expression (37). Therefore, we hypothesized that high GLU would also affect the expression of IGF-1 family members in

human DPCs that underwent apoptosis and differentiation. Our results revealed that 25 and 50 mM GLU markedly decreased

Figure 6. Effect of insulin-like growth factor-1 (IGF-1) on the expression of mineralization-related proteins under high glucose (GLU) conditions. The expression levels of mineralization-related proteins, including osteocalcin (OCN), osteonectin (ON), osteopontin (OPN), dentin sialoprotein (DSP) and dentin matrix protein-1 (DMP-1) were examined by western blot analysis 7 days after differentiation. ** $P < 0.01$ vs. control group; * $P < 0.05$ and ## $P < 0.01$ vs. odontoblastic induction medium (OM) group; & $P < 0.05$ and && $P < 0.01$ vs. OM+25 mM GLU group.

the expression levels of IGF-1, IGF-1R, IGFBP1 and IGFBP3 in DPCs that were cultured in normal DMEM for 24 h or in OM after 7 days of differentiation. Additionally, we found that 25 mM GLU significantly decreased the IGF-1, IGF-1R, IGFBP1 and IGFBP3 levels from day 3 or 7 after differentiation.

IGF-1 binds to and activates the receptor IGF-1R and thus promotes cell proliferation and survival (38). Zhang *et al* reported that IGF-1 attenuated high fat diet-induced mitochondrial damage, myocardial contraction dysfunction and cardiomyocyte apoptosis (39). As expected, our results revealed that IGF-1 reversed the effects of high GLU on cell proliferation and protected the DPCs against apoptosis, suggesting the protective effects of IGF-1 in DPCs under conditions of high GLU.

It is well known that high GLU inhibits the osteoblast differentiation of MC3T3-E1 cells (40). IGF-1 has been demonstrated to promote the proliferation and differentiation of osteoblasts and chondrocytes *in vitro* (41). However, the effects of IGF-1 on the differentiation of human DPCs under high GLU conditions remain unknown. The process of odontoblastic differentiation involves multiple proteins (1). OCN is an important differentiation marker that is found in odontoblasts, dentine matrix and bone matrix. It is associated with mineralization and matrix deposition (42). ON is a major non-collagenous protein that is responsible for bone and dentin mineralization (43). OPN, a secreted glycoposphoprotein, is an odontoblastic marker for early differentiation (44). DSPP is a collagenous extracellular matrix (ECM) protein that partici-

pates in dentin mineralization. It can be cleaved into DSP and dentin phosphoprotein (DPP) (45). DMP-1 is also correlated with dentin formation and mineralization (46). In our study, the differentiation of DPCs into odontoblastic cells was evaluated by measuring ALP activity, mineralization and the levels of mineralization-associated proteins (OCN, ON, OPN, DSP and DMP-1). We provide similar findings that 25 mM GLU markedly suppressed the activity of ALP and decreased the mineralized matrix deposition in primary human DPCs that underwent differentiation. Furthermore, 25 mM GLU significantly decreased OCN, ON, OPN, DSP and DMP-1 expression in DPCs during differentiation. These results indicate that high GLU inhibits the odontoblastic differentiation of DPCs. However, IGF-1 restored ALP activity and mineralization in the DPCs, suggesting that IGF-1 attenuates the effects of high GLU and promotes the odontoblastic differentiation of DPCs under high GLU conditions by increasing the expression levels of mineralization-related proteins.

In conclusion, the findings of the present study suggest that IGF-1 promotes the survival and odontoblastic differentiation of DPCs, and protects the cells against apoptosis in a high GLU environment.

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