Role and mechanism of bcl-2 and bax in high glucose-mediated apoptosis of human periodontal ligament cells

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Abstract

BACKGROUND: High glucose can trigger the apoptosis of human periodontal ligament cells, in which, whether bcl-2 and bax are started? How do they work? There is still no relevant report.

OBJECTIVE: To investigate the effect of high glucose on bcl-2 and bax mRNA expression as well as apoptosis in human periodontal ligament cells in vitro.

METHODS: Human periodontal ligament cells were primarily cultured and identified. Then, cells at 5–8 passages were selected in this experiment. Cells were treated with 5.5 mmol/L (control group) and 25 mmol/L (high glucose group) glucose for 24 and 48 hours. Cell apoptosis was determined by Hoechst 33258 staining and the expression of bcl-2 and bax mRNA was detected by Real-time PCR.

RESULTS AND CONCLUSION: High glucose (25 mmol/L) could induce the apoptosis of human periodontal ligament cells and increase bcl-2 and bax significantly. The bcl-2/bax ratio showed a more significant decline in the high glucose group compared with the control group (P < 0.05). These findings indicate that high glucose can induce the apoptosis of human periodontal ligament cells, in which, Bcl-2 family plays an important role.

Subject headings: periodontal ligament; diabetes mellitus; apoptosis; genes, bcl-2

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INTRODUCTION

Periodontal diseases refer to a series of diseases affecting the health of periodontal tissue, which are caused by periodontal pathogens such as Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia, and Aggregatibacter actinomycetemcomitans. These infections can lead to oral tissue destruction, tooth dislocation and loose of tooth[1]. Currently, patients with severe periodontitis in the population accounts for 10%–15%, even up to 30%[2]. Periodontitis is a common human oral disease, and also the main reason for adult tooth loss.

Susceptibility to periodontitis is dramatically varying in the population, depending on host’s reactions to periodontal pathogens. Although periodontal disease is directly caused by bacterial plaque-induced inflammation, the host health and genetic factors have a great impact on the clinical manifestations and progression of periodontal disease[3].

Periodontal ligament cells are the most common periodontal ligament cells to continuously form new primary fibers and cementum as well as to remodel the alveolar bone. Periodontal ligament cells show good functions in tissue regeneration and functional recovery, which play a vital role in inflammatory periodontal tissue[4].

Diabetes mellitus is a metabolic disease characterized by high blood glucose. Persistent high blood glucose can lead to chronic damage and dysfunction to a variety of tissues[5–6]. There is a close tie between periodontal disease and diabetes mellitus, and the former one is regarded as the sixth largest periodontal complications of diabetes mellitus[7–8]. On the one hand, diabetic patients relatively have high prevalence of periodontal disease that is difficult to treat[9–10]; on the other hand, if glucose metabolism has been effectively controlled, the improvement in periodontal disease is obvious. Outcome measures for two diseases also have some connection, for example, correlation between...
glycated hemoglobin and periodontal indices and correlation between plasma lipid peroxide (oxidative stress index) and periodontal markers\textsuperscript{(11-12)}. Its mechanism may be that high sugar inhibits periodontal ligament cell proliferation, and induces cell apoptosis as well as impacts related growth factors related.

Apoptosis is an active and ordered cell death process under gene control, which is a basic physiological activity for multicellular organisms to remove damaged and harmful cells\textsuperscript{(13)}. If this physiological activity is abnormal, many diseases can occur. Bcl-2 family is a gene family controlling cell apoptosis\textsuperscript{(14-15)}, which can be divided into anti-apoptotic genes (such as bcl-2) and pro-apoptotic genes (such as bax). High glucose can induce apoptosis of human periodontal ligament cells, in which, whether bcl-2 and bax can be started? How do they work? There is no relevant report. This study aimed to explore the mechanism of action of bcl-2 and bax by stimulating human periodontal ligament cells by high glucose.

MATERIALS AND METHODS

Design
A single sample observation.

Time and setting
The experiment was completed in the Central Laboratory of the Affiliated Hospital of Qingdao University Medical School from January to June 2014.

Materials
Reagents and equipments for bcl-2 and bax in high glucose-mediated apoptosis of human periodontal ligament cells are as follows:

<table>
<thead>
<tr>
<th>Reagents and instruments:</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent and instrument</td>
<td></td>
</tr>
<tr>
<td>Fetal bovine serum</td>
<td>Gibco, USA</td>
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<tr>
<td>αMEM, DMEM culture medium, trypsin</td>
<td>Hyclone, USA</td>
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<tr>
<td>Hoechst staining kit</td>
<td>Beyotime Biotechnology, Hangzhou, China</td>
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<tr>
<td>FastStart Essential DNA Green Master Kit</td>
<td>Roche, Switzerland</td>
</tr>
<tr>
<td>3111 type incubator</td>
<td>ThermoForma, USA</td>
</tr>
<tr>
<td>DMIL Inverted phase contrast microscope</td>
<td>Leica, Germany</td>
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<tr>
<td>LightCycler 96 Real-time Fluorogenic Quantitative PCR</td>
<td>Roche, Switzerland</td>
</tr>
</tbody>
</table>

Methods

Culture and identification of human periodontal ligament cells
Tissue explant method and enzymatic digestion method were used\textsuperscript{(16)}. Specimens were provided by the Department of Stomatolgy, Qingdao Municipal Hospital, and informed consent was obtained from children and their parents. Health premolar teeth were extracted from 11-14-year-old patients because of orthodontic removal, and placed immediately into a precooled sterile αMEM medium. The subsequent operations were carried out in a super clean bench. The teeth were rinsed with 0.01 mol/L PBS containing 1 000 U/L penicilllin and 1 000 U/L streptomycin. A sterile scalpel blade was used to scrape the periodontium at middle 1/3 of the root, and the specimens were placed into PBS containing 0.3 g/L I collagenase at 37 °C water bath for 0.5 hour oscillation digestion, gently pipetted and centrifuged (1 000 r/min×5 minutes). After removal of the supernatant, the cell pellet was cultured in αMEM medium containing 20% fetal bovine serum, resuspended, and centrifuged (1 000 r/min×5 minutes). After supernatant removal, a small amount of αMEM medium containing 20% fetal bovine serum was added and gently pipetted. The suspension (tissue blocks containing a single cell and undigested ones) was seeded in culture flasks, and there was an interval of about 5 mm between tissue blocks. The flasks were reversed, and 3 mL of α-MEM medium containing 20% fetal bovine serum, 100 U/L penicillin and 100 U/L streptomycin was added. Then, the culture flasks were placed in a CO2 thermostat incubator (5% CO2, 100% humidity, 37 °C) in static culture. Four hours later, the culture flasks were reversed again, and the bottom was downward. The medium was exchanged every 4 days, and under the inverted microscope, cell extravasation and growth were observed. When the tissue block was covered with cells and 30% cells were in the culture bottles, cells were subcultured. Immunohistochemical staining was employed to determine cell source, and cells at passages 5-8 were used in the experiment.

Hoechst33258 fluorescent staining of high glucose and lipopolysaccharide effects on human periodontal ligament cell apoptosis
According to the previous references and pre-experimental results, there were two groups in the study, physiological control group with a glucose concentration of 5.5 mmol/L, high glucose group with 25 mmol/L of DMEM medium as intervention reagent\textsuperscript{(17-18)}. Cells at a density of 1×10\textsuperscript{5} per well were seeded into 24-well culture plates, and αMEM culture medium containing 10% fetal bovine serum was added. When the cell density reached about 70%, the culture medium was removed and cells were subjected to starvation culture in serum-free αMEM medium for 12 hours. According to the grouping, two kinds of interventional reagents were added, respectively, three wells in each group, for 24 and 48 hours. After that, the culture medium was aspirated, 0.5 mL fixative per well was added. After 10 minutes, the fixative was removed and the plates were rinsed with PBS twice, 3 minutes once. Then, 0.5 mL Hoechst33258 dye liquor was added for 5 minutes dyeing, and the plates were shaken several times by hand. The dye liquor was aspirated dye, and the plates were washed with PBS twice, observed and photodet under the fluorescence microscope. The excitation wavelength was 350 nm, and the emission wavelength was 460 nm.
Real-time PCR detection of bax and bcl-2 expressions

Two-step RT-PCR was adopted in the study. Hoechst33258 fluorescence staining was done as above. After that, 0.5 mL Trizol per well was added at 15-30 °C standing for 5 minutes, so nucleoprotein could be fully dissociated. After 0.1 mL chloroform was added, the plates were fully shaken vigorously for 15 seconds, standing at 15-30 °C for 2-3 minutes, followed by 12 000 r/min centrifugation at 4 °C for 15 minutes. Centrifuged samples were layered, and RNA was found in the upper aqueous phase. The supernatant was transferred to a new tube, added with 0.3 mL isopropanol, mixed by inversion and stood at 15-30 °C for 10 minutes. Then, some gelatinous precipitates appeared on the bottom of the tube, namely RNA. The RNA samples were centrifuged at 4 °C at 12 000 r/min for 10 minutes, and the supernatant was discarded. The RNA samples were dried in 55°C ethanol containing 0.1% DEPC and mixed gently. After 12 000 r/min centrifugation for 5 minutes at 4 °C, the supernatant was removed. The RNA samples were dried 10 minutes, and dissolved in 30 μL of DEPC at 55-60 °C for 10 minutes. Afterwards, the A_{260}/A_{280} ratio was measured. Transcriptor First Strand cDNA Synthesis Kit was used for RNA reverse transcription and synthesis of cDNA. Primer design based on the coding region is shown in Table 1.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence(5’-3’)</th>
<th>Size (bp)</th>
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<tr>
<td>bax</td>
<td>Forward: AGT GGC AGC TGA CAT GTT TT</td>
<td>152</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGA GGA AGT CCA ATG TCC AG</td>
<td></td>
</tr>
<tr>
<td>bcl-2</td>
<td>Forward: ATG TGT GTG AGC GTC AAC C</td>
<td>196</td>
</tr>
<tr>
<td></td>
<td>Reverse: TGA GCA GAG TCT TCA GAG ACA GCC</td>
<td></td>
</tr>
<tr>
<td>GADPH</td>
<td>Forward: TCA TGG GTG TGA ACC ATG AGA A</td>
<td>146</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGC ATG GAC TGT GGT CAT GAG</td>
<td></td>
</tr>
</tbody>
</table>

RESULTS

Morphology and sources of human periodontal ligament cells

After 4-7 days of culture, human periodontal ligament cells were found swim from the surrounding tissue block. Cells were extended to form a stellate or fusiform shape and interconnected to form a mesh. Sporadic cells were visible in an empty area with no tissue block. With the increase of cell density, cells were spindle-shaped and exhibited a tissue block-centered arrangement in a radial appearance. Immunohistochemical staining showed that the anti-vimentin expression polyclonal antibody expressed positively, the cytoplasm was brown in color, and anti-keratin expression was negative (Figure 1).

Results of Hoechst33258 staining

Nuclear condensation, fragmentation, and appearance of apoptotic bodies are important characteristics of cell apoptosis. Under the fluorescence microscope, apoptotic cells had stained and dense nuclei in the color of bright white, and normal cells, round or oval, showed a uniform blue fluorescence with clear nucleus boundaries. Apoptotic cells were rare in the normal control group, while apoptotic cells, even apoptotic bodies, were visible in the high glucose group. The number of apoptotic cells or apoptotic bodies was increased significantly in the high glucose group with time (Figure 2).

mRNA expression of bcl-2 and bax

Compared with the control group (5.5 mmol/L), the bcl-2 and bax expression levels in human periodontal ligament cells were significantly increased in the high glucose group (25 mmol/L) at 24 and 48 hours (P < 0.05). The bcl-2/bax ratio, however, was significantly lower in the high glucose group than in the control group (P < 0.05; Figure 3).

DISCUSSION

Due to the frequent physiological activities, such as chew and language, periodontal tissue remodeling is very active. Periodontal ligament cells are one of the most important cells in the periodontal ligament tissue, which play an ignored role in periodontal tissue regeneration and new attachment formation. Under the interference of some virulence factors, the biological activity of human periodontal ligament cells is inhibited, thereby affecting the normal function of the periodontal ligament that can eventually lead to periodontal diseases.

Clinical and epidemiological surveys have found a high incidence of periodontitis in diabetic patients, with serious condition and rapid progress; on the other hand, after the effective control of blood glucose, periodontal disease has also been improved significantly. In basic research, the effects of high glucose on human periodontal ligament cells are manifold, mainly in terms of proliferation, collagen synthesis, differentiation, and host immune response.

Cell proliferation is the basis of biological growth,
development and reproduction. High glucose can remarkably inhibit the in vitro proliferation of human periodontal ligament cells. Kim et al. found that when the glucose concentration is 25 mmol/L, cell proliferation can be suppressed. Basic fibroblast growth factor is a heparin-bonded peptide directly involved in cell division and proliferation to promote blood vessel growth and development. Basic fibroblast growth factor is associated with the proliferation and migration of human periodontal ligament cells in vitro, high glucose inhibits secretion of basic fibroblast growth factor. Ohgi and co-workers discovered that basic fibroblast growth factor expression is decreased with the increasing glucose concentration (5.5 mmol/L and 20 mmol/L). Experimental findings indicate that high glucose can weaken the cell division and proliferation by inhibition of basic fibroblast growth factor.

Collagen is the main component of the periodontal ligament, which guarantees the structure stability, normal function and physiologic regeneration of the periodontal tissue. High glucose inhibits collagen secretion. Chang et al. interfered periodontal ligament cells with high glucose and lipopolysaccharide, and they found that only high-concentration glucose could significantly inhibit both type I and type IV collagen expression levels.

Under the stimulation of certain factors, periodontal ligament cells with a variety of biological functions can differentiate into osteoblast-like cells. High glucose can reduce the osteogenesis and mineralization capacity of periodontal ligament cells. Kim et al. observed when the periodontal ligament stem cells were exposed to high glucose (30 mmol/L), a reduction was found in osteoblast activity. Scleraxis is a member of Twist subfamily in cell-specific basic helix-loop-helix transcription factor, closely related to the differentiation of connective tissue. High glucose can increase mRNA expression of Scleraxis but inhibit osteogenic differentiation of periodontal ligament cells. Yuan et al. detected Scleraxis mRNA expression in human periodontal ligament cells was significantly increased under high-glucose conditions, and alkaline phosphatase activity decreased. Experimental findings indicate that high glucose raises Scleraxis expression, thereby inhibiting osteogenic differentiation. Kim et al. found that, under high-glucose conditions, the mineralization percentage of periodontal ligament cells was lower than that under normal conditions.

Bacterial activity-activated host immune inflammatory response is considered to be an important part of soft and hard tissue damage in periodontal diseases. Some inflammatory cytokines, including interleukin-1, interleukin-6 and tumor necrosis factor-α, may induce damage to the connective tissue and alveolar bone. High expressions of these inflammatory cytokines are found in the gingival sulcus fluid of patients with gingivitis and periodontitis, suggesting that elevated levels of these cytokines may exert an important role in periodontal tissue damage.

Numerous studies have indicated that the aggravation of diabetes mellitus can cause an increase in in vivo inflammatory factors and endotoxin levels as well as host responses, and then worsen chronic periodontitis. High glucose increases gene expression of inflammatory factors. A study from Fang found that high glucose itself can either elevate interleukin-1B, interleukin-6, tumor necrosis factor-α gene expressions, or enhance the advanced glycation end products. Co-existence of high glucose and inflammatory factors can lead to more severe local damage to the periodontal tissue.

Toll-like receptors (TLR) are a class of important protein molecules involved in nonspecific immunity, but a bridge between non-specific and specific immunities. TLR4 exists in fibroblasts, macrophages and epithelial cells from the periodontal tissue. It is speculated that high blood glucose can contribute to the expression of these receptors in the periodontal tissues and improve the content of the corresponding ligand, thus accumulating ligand components. Studies have shown that, either chemical or mechanical periodontal treatments can improve blood glucose and lipid levels. Chang et al. found that high glucose can enhance the effect of lipopolysaccharide on the expression of TLR2 in periodontal ligament cells.

Apoptosis is a programmed cell death process stimulated by gene regulation under certain conditions. In normal physiological activities, apoptosis plays an active role in clearing aging and damaged cells, responding to environmental changes in vitro and in vivo, maintaining the homeostasis in the body. This imbalanced microenvironment may be associated with many diseases. Increasing studies have suggested that cell apoptosis is involved in the occurrence and development of periodontitis. High glucose can function normally by inducing apoptosis of periodontal ligament cells. Jiaqiang et al. found that when 25 mmol/L glucose can induce apoptosis in human periodontal ligament cells in a concentration-dependent manner.

Hoechst33258 staining is a common method for observation of apoptotic morphology, characterized as intuitive observation of apoptosis and simple and fast operation, thereby determining whether the cell apoptosis occurs and providing a good reference for subsequent experiments. After 24 hours of high glucose stimulation, periodontal ligament cells can be found in an increase of apoptotic cells, chromatin condensation phenomenon occurs; and apoptotic bodies appear after 48 hours.

Mitochondria are the central link in the regulation of apoptosis, and the mechanism is associated with Bcl-2 protein family interactions, changes in the mitochondrial membrane, intracellular Ca2+ overload and oxygen free radicals. Bcl-2 family proteins are located in the mitochondrial membrane and can lead to apoptosis directly or by activating Caspase pathway, which can be divided.
into anti-apoptotic genes (such as \textit{bcl-2}) and pro-apoptotic genes (such as \textit{bax}). Expressions and interaction of anti-apoptotic genes and pro-apoptotic genes determine whether cell survival or apoptosis occurs. During apoptosis process, both of these two genes will be increased, which reflects cell self-healing and self-protesting ability. As the intracellular \textit{bcl-2/bax} ratio continues to decrease, to study the \textit{bcl-2/bax} ratio is of greater significance\cite{s34}. In the present study, the expressions of \textit{bcl-2} and \textit{bax} were both higher in the experimental group than the control group; moreover, their expressions were higher at 48 hours than at 24 hours, indicating there are two kinds of reactive homeostasis—cell proliferation and apoptosis\cite{s35}. By calculating the \textit{bcl-2/bax} ratio, we found that the homeostasis with the enhancement of interfering factors was tilted toward the apoptosis, suggesting that pro-apoptotic genes were occupying a leading position.

In summary, high glucose can promote apoptosis in human periodontal ligament cells, during which, Bcl-2 family plays an important role. Jia \textit{et al} \cite{s32} found that Caspase-3 inhibitor z-DEVD-fmk can partially inhibit high glucose-induced apoptosis of periodontal ligament cells. Therefore, the high glucose-induced apoptosis in human periodontal ligament cells is considered to be mediated by Caspase signaling pathways in part. On the whole, high glucose is speculated to start the mitochondrial apoptosis pathway.

Apoptosis in human periodontal ligament cells is prone to occurring in diabetic patients or under high glucose conditions. Moreover, these patients have more frequent episodes of periodontal diseases and worse healing. In response to these situations, two aspects should be considered at least. For patients: (1) to reduce the intake of low-nutrient and
high-glucose foods is the basic measure to reduce these risk factors. Nutritious, high-fiber foods such as fruits and vegetables can keep a lot of health benefits. (2) To improve chewing performance. Full or partial removable dentures cannot be comparable to nature teeth in terms of chewing efficiency; however, the fixed partial dentures and implant dentures are helpful to restore chewing performance and improve diet structure. For doctors: the oral complications of diabetes mellitus mainly include periodontal disease, candidiasis, burning mouth syndrome, and acute oral infections. All these diseases are basically to be found and treated in dental treatment. Therefore, the dentists plays a fundamental role in the diagnosis, treatment and control of metabolic patients. In the future, a higher level of multidisciplinary cooperation between dentists and endocrinologists is required to implement the treatment of metabolic syndrome and its complications.

REFERENCES


高糖介导牙周膜细胞凋亡中 bcl-2、bax 的作用及机制

任伟伟，陈书兰，仇静，卢恕来，刘海蓉，山东省青岛市口腔科，山东省青岛市 266071；3 青岛大学医学院附属医院中心实验室，山东省青岛市 266555

摘要
背景：高糖环境下人牙周膜细胞会发生凋亡。其中 bcl-2、bax 作用及机制
目的：应用不同浓度葡萄糖影响人牙周膜细胞，观察细胞凋亡及 bcl-2、bax 基因表达变化
方法：1. 实验结果表明，Hoechst 33258 免疫荧光染色观察人牙周膜细胞凋亡；RT-PCR 显示 5.5 mmol/L 葡萄糖 bcl-2、bax 表达显著高于对照组，其中 bcl-2/bax 比值显著降低。结果说明高糖可增加人牙周膜细胞凋亡，bcl-2/bax 基因表达显著增加。2. Real-time PCR 检测了人牙周膜细胞高糖诱导下 bcl-2、bax 表达。
结果与结论：bcl-2/bax 作用机制与细胞凋亡密切相关。高糖介导下人牙周膜细胞发生凋亡，其中 bcl-2、bax 比值显著降低，对细胞凋亡有抑制作用。高糖可增加人牙周膜细胞凋亡，bcl-2/bax 基因表达显著增加。

关键词：高糖；人牙周膜细胞；凋亡；bcl-2；bax

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