



Effects of high glucose on cellular activity of periodontal ligament cells in vitro

Hyun Sook Kim^a, Jin Woo Park^a, Shin Il Yeo^a, Byung Ju Choi^b, Jo Young Suh^{a,*}

^a Department of Periodontology, College of Dentistry, Kyungpook National University,
188-1 Sanduk 2Ga, Jung-Gu, Daegu 702-412, South Korea

^b Department of Dental Pharmacology, College of Dentistry, Kyungpook National University,
188-1 Sanduk 2Ga, Jung-Gu, Daegu 702-412, South Korea

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Abstract

Periodontal ligament (PDL) cells are the most important cells in the healing of wounds and the regeneration of periodontal tissues. The response of PDL cells regarding cellular activity to high glucose concentration levels could be the key in understanding the events associated with the dental care of brittle diabetes. We studied the effect of high glucose concentration levels on the cellular activity of PDL cells from five non-diabetic patients in vitro. PDL cells were cultured for 14 days in a normal glucose medium (1100 mg/l of glucose) or in a high glucose medium (4500 mg/l of glucose) and a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay for cellular viability was also performed. In order to evaluate the differentiation of PDL cells to osteoblast-like cells, mineralized nodule formation was induced with supplemented media containing 50 µg/ml of ascorbic acid, 10 mM of β-glycerophosphate and 100 nM of dexamethasone for 21 days. High glucose significantly inhibited the proliferation of PDL cells and reduced the optic density of the MTT assay. Concerning the mineralized nodule formation, the percentage of the calcified area to the total culture dish of PDL cells in high glucose level was lower than that in the normal glucose medium. In conclusion, high glucose inhibits the proliferation and differentiation of PDL cells. The data provide an explanation for the delayed periodontal regeneration and healing in diabetic patients.

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1. Introduction

Periodontal ligament (PDL) is the primary target of inflammatory tissue destruction in periodontal disease. The normal PDL includes osteoblasts, osteoclasts, fibroblasts, epithelial cell rests of Malassez, macrophages, undifferentiated mesenchymal cells, neural elements, endothelial cells, and cementoblasts. Fibro-

blasts are the predominant cells in the PDL. The role of the PDL cells in tissue regeneration is well characterized [1,2]. Only PDL cells can form new attachments including new cementum with inserting collagen fibers [3,4]. Several studies have also demonstrated that PDL cells are not homogenous but may consist of different subpopulations of unique phenotypes and distinct functional activities [5,6]. Since PDL cells are always exposed to microbial challenges and subsequent host immune reactions, one might expect that the high glucose levels in diabetic patients could alter the regenerative capacity of the PDL cells against periodontal breakdown.

* Corresponding author. Tel.: +82 53 420 5955;
fax: +82 53 427 3263.

E-mail address: jysuh@knu.ac.kr (J.Y. Suh).

Many reports have supported the high prevalence of periodontal disease among diabetic patients [7–10]. Although there are several hypotheses which explain the possibility of this occurring, the exact mechanism by which diabetes interferes with oral health remains unclear [11,12]. Ohgi and Johnson [13] searched whether high glucose modulates the growth of gingival fibroblasts and PDL cells, *in vitro*, by using hemocytometer. They showed that gingival fibroblasts proliferated or were unaffected by glucose, while the growth of PDL cells was inhibited. Nishimura et al. [11,12] showed that the fibronectin receptor expression was much higher in cells cultured in a high glucose medium than in periodontal ligament cells cultured in a normal glucose medium. The over-expression of the fibronectin receptor resulted in a suppressed chemotactic response of these cells to a platelet-derived growth factor.

Studies focusing on the characterization of the PDL cells, *in vitro*, indicate that these cells have osteoblastic-like properties and the PDL cells have the capacity to produce mineralized nodules, *in vitro*, under mineralized media which include ascorbic acid, β -glycerophosphate and dexamethasone [5,14–16]. Thus, high glucose concentration levels probably influence the mineralization and differentiation of PDL cells to osteoblast-like cells.

The direct effect of high glucose on the proliferation and differentiation of human PDL cells, however, is less well established. In this study, we prepared primary cultures of PDL cells from the extracted teeth of patients and investigated the effect of high glucose on the PDL cells.

2. Material and methods

2.1. Cell Isolation and culture

The PDL tissues were obtained from five non-diabetic patients. The periodontal ligaments of premolar teeth were extracted for orthodontic reasons. After extraction, the teeth were placed and rinsed in a biopsy media, Dulbecco's modified Eagles media (DMEM) with 500 U/ml penicillin and 500 μ g/ml of streptomycin. After rinsing, only the periodontal ligament attached to the middle third of the root was removed with a curette. The PDL tissues were cut into small pieces and placed in small culture dishes. The tissues were incubated in a biopsy medium at 37 °C, 5% CO₂, and 95% humidity. The medium was replaced with a culture medium (DMEM with 10% FBS, 100 U/ml of penicillin and 100 μ g/ml of streptomycin). After reaching confluence, the cells were passaged with 0.25% trypsin/0.1% ethylene diaminetetraacetic acid (EDTA). Cells between the 3rd passage and 8th passage were used in the following studies. In order to determine the effect

of high glucose, we used DMEM containing 1100 mg/l of glucose (normal glucose) and DMEM containing 4500 mg/l of glucose (high glucose) [11,12]. The osmolarity of normal glucose and high glucose was 313 and 339 mOsm/kg, respectively.

2.2. Cell proliferation

Five different non-diabetic patients' PDL cells were used for proliferation. The PDL cells were seeded at a density of 2×10^4 cells/well in a 2 ml normal glucose DMEM, supplemented with 10% FBS in a 12-well plate. After 24 h, the control group medium and experimental group medium was changed with the same conditioned medium and high glucose medium (4500 mg/l of glucose), respectively, supplemented with 10% FBS. During each experimental period, the culture media were changed every 3 days. The numbers of PDL cells were determined by counting the six wells per each group with a hemocytometer at various times (1, 4, 7, 10, 14 days) during the incubation. Statistical analyses were performed with Student's *t*-test.

2.3. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay for cellular viability

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to evaluate cell viability [17]. The MTT was transformed by mitochondrial hydrogenases into formazan, enabling mitochondrial activity and cell viability to be assessed. Five samples per group were evaluated. The PDL cells were seeded at a density of 1×10^4 cells/well on the 96-well plate.

On days 1, 4, and 7, 50 μ l of pre-warmed (37 °C) MTT solution was added to each well and cultured for 3 h. Then, the reaction was stopped by addition of 200 μ l of dimethyl sulfoxide (DMSO) and 50 μ l of glycine buffer to each well. The solution was then transferred to new wells and the optical density of the solution in each well was measured at a wavelength of 570 nm using an ELISA plate reader (Precision Microplate Reader, Molecular Devices, USA) [18]. A Student's *t*-test was used to evaluate the differences between cell viability of the normal and high glucose concentration levels.

2.4. Mineralized nodule formation

The PDL cells were plated at a density of 1×10^6 cells in a 100 mm Type I collagen-coated Petri dish and incubated in DMEM (containing 1100 mg/l glucose) supplemented with 10% FBS, 100 U/ml of penicillin and 100 μ g/ml of streptomycin. Several studies showed that PDL cells formed mineralized nodules when they were cultured in supplemented medium containing 50 μ g/ml of ascorbic acid, 10 mM of β -glycerophosphate, and 100 nM of dexamethasone [5,14,16]. Cells were grown until confluent which was designated as day 0, and nodule formation was induced by the addition of 50 μ g/ml of ascorbic acid, 10 mM of β -glycerophosphate and 100 nM of

Table 1
Growth changes of PDL cells

	Day 1	Day 4	Day 7	Day 10	Day 14
PDL 1					
High	5.04 ± 0.21	15.45 ± 0.50*	24.96 ± 1.44*	39.06 ± 2.59*	37.10 ± 3.49*
Normal	5.01 ± 0.24	20.07 ± 0.98	28.35 ± 0.81	44.87 ± 1.86	50.26 ± 0.70
PDL 2					
High	3.71 ± 0.59	16.59 ± 1.42	19.08 ± 1.37	33.88 ± 3.24*	63.35 ± 2.10*
Normal	3.54 ± 0.54	18.73 ± 1.39	20.63 ± 1.28	44.94 ± 5.76	70.70 ± 2.91
PDL 3					
High	2.70 ± 0.45*	11.48 ± 1.07*	30.13 ± 1.90*	51.25 ± 2.36	58.25 ± 4.57*
Normal	3.68 ± 0.38	14.08 ± 1.30	35.65 ± 2.22	55.25 ± 3.30	67.50 ± 1.29
PDL 4					
High	2.48 ± 0.22*	19.25 ± 0.93*	29.0 ± 1.68*	54.0 ± 1.63*	57.7 ± 6.85
Normal	2.95 ± 0.13	24.43 ± 3.41	32.7 ± 2.45	59.5 ± 1.91	66.0 ± 5.89
PDL 5					
High	2.4 ± 0.22*	10.9 ± 0.47*	20.6 ± 1.48	40.3 ± 1.26*	48.0 ± 2.45*
Normal	3.15 ± 0.24	12.8 ± 0.22	22.8 ± 2.38	46.3 ± 2.06	57.0 ± 1.83

Numeric data represents the mean ± S.D. of cell numbers ($\times 10^4$). PDL, periodontal ligament; high, high glucose group; normal, normal glucose group. * $P < 0.05$: compared to normal group.

dexamethasone to the medium. The dishes were randomly divided into two treatment groups: 1100 mg/l glucose (normal) or 4500 mg/l glucose (high). The media were changed every 2–3 days. Four dishes of each group were randomly selected at days 14 and 21 and stained for mineralization nodules with Alizarin Red S, as previously described [19]. To determine mineralization, the percentages of calcified areas to total areas per dish and per visual field (randomly selected three per dish) were analyzed by using an image analysis program I-solution (IM Technology, Korea). The number of nodules was also determined at a 40 \times magnification from three randomly selected visual fields per dish (a total of 12 fields per group) from two independent experiments. Statistical analysis were performed with Student's *t*-test.

3. Results

3.1. Cell proliferation

In Table 1, the growth patterns of five different PDL cells are shown. The data represent the growth rate of the PDL cell populations cultured as monolayers in the presence of a high concentration of glucose (4500 mg/l of glucose) and normal glucose (1100 mg/l of glucose). The PDL cells 1 in high glucose medium started to show a significantly reduced proliferation rate compared to the PDL cells in the normal glucose medium on day 4 and the statistical difference remained until day 14 ($P < 0.05$). The PDL cells 2 in high glucose medium had statistically lower cell numbers than the PDL cells in normal glucose medium on days 10 and 14 ($P < 0.05$). The PDL cells 3, 4, and 5 in high glucose

medium showed a similar tendency of the reduced proliferation rate compared to the PDL cells in the normal glucose medium from day 1 to day 24.

3.2. MTT assay for cellular viability

The PDL cells from five healthy periodontium were exposed to 1100 and 4500 mg/l of glucose. On day 1 and day 7, the MTT assay indicated no significant differences in the cell viability between the high and normal glucose groups for any of the cell types (data not shown). On day 4,

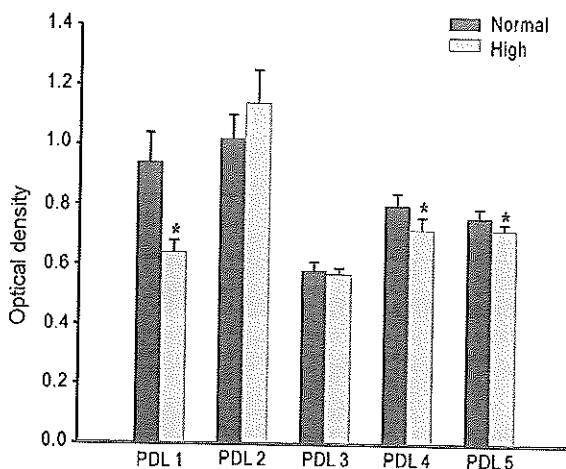


Fig. 1. Cellular viability of PDL cells. Optical density was measured after incubation of PDL cells from five different individuals (PDL 1, PDL 2, PDL 3, PDL 4, PDL 5) with normal glucose medium (normal) or high glucose medium (high). *Statistically significant between normal medium and high glucose medium: $P < 0.05$.

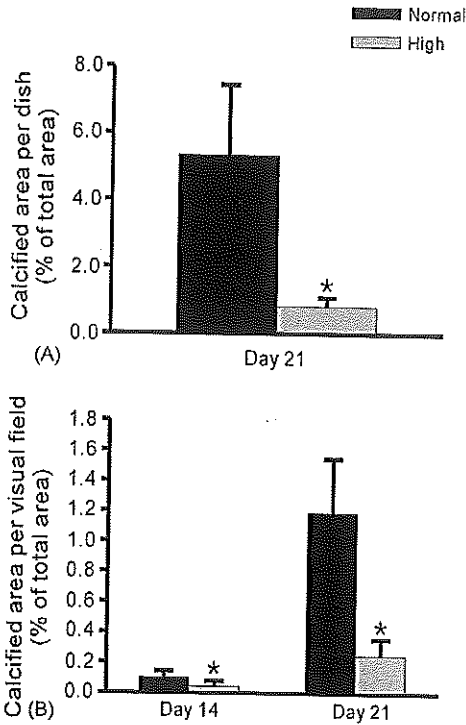


Fig. 2. Effect of high glucose on the mineralization. For 14 days and/or 21 days incubation period. Percentages of calcified area to total area per dish (A) and per visual field (B) were analyzed. Data are shown as the mean \pm S.D. of 12 determinations. *Significant differences from normal value with $P < 0.05$.

PDL cells 1, 4, and 5 in the high glucose medium showed a significantly decreased optical density ($P < 0.05$, Fig. 1).

3.3. Mineralized nodule formation

The mineralized nodule formation is the most prominent characteristic of osteoblast-like cells. The

percentage of the calcified area and the numbers of nodules were analyzed to evaluate the characteristics of osteoblast-like cells. On days 14 and 21, the culture dishes were stained with Alizarin Red S. The PDL cells incubated in DMEM, supplemented with a mineralized medium, developed nodules both in the normal and high glucose groups (Figs. 2 and 3). Small-mineralized nodules started to develop on day 14. On day 21, mineralization was distinct for both group, the nodule size increased and more nodules formed. At that time, the PDL cells in the normal glucose medium developed further and the nodules were bigger than the PDL cells in the high glucose medium. The percentage of the calcified area to total area per culture dish and the percentage of the calcified area to visual field area per randomly selected visual field (three visual fields per dish) were analyzed using an image analysis program, I-solution (IM Technology, Korea). The mean percentage of the calcified area to total culture dish of the PDL cells in the high glucose concentration levels was lower than that in the normal glucose medium on day 21 ($P < 0.05$). There was no statistical difference in terms of the percentages of calcified area to total area between the experimental and control groups on day 14. High glucose, however, decreased the calcified areas on the magnified visual field. Also, glucose decreased the calcified area per visual field on day 21.

4. Discussion

Fibroblasts perform many functions such maintaining tissue architecture, synthesizing extracellular matrix, and participating in intercellular communication. Fibroblasts are the most predominant in periodontal ligament connective tissue and occupy about half

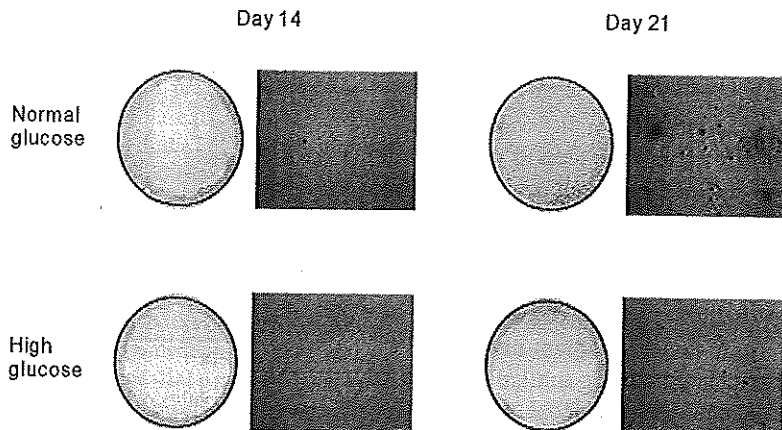


Fig. 3. Alizarin Red S staining of the mineralized nodules cultured for 14 days and 21 days with normal glucose medium and with high glucose medium. Circular and square photographs show PDL cells at culture dish (\varnothing : 100 mm) and visual field (40 \times magnification), respectively.

of the PDL. The PDL cells synthesize various extracellular matrix proteins and proteolyse old collagen fibril. Also, PDL cells can be differentiated into osteoblast-like and cementoblast-like cells that can produce and restore bone and cementum. Thus, normal cellular activity of PDL cells is essential for periodontal regeneration [5,14,20].

Our results clearly demonstrate a decrease in the proliferative capacity of PDL cells in high glucose concentration level. Also, the difference in the numbers of cells between high glucose and low glucose media increased with time. Also, the decreased optical densities of an MTT assay were seen on the PDL cells in a high glucose medium, and that means reduced cell viability. These findings suggest that sustained high glucose concentration amounts alone inhibit PDL cell proliferation.

Our results suggest that PDL cells respond in the same way other cells do regarding growth rate in high glucose concentration amounts. Turner and Bierman [21] and Hehenberger and Hansson [22] stated that glucose was important for cell proliferation. They have shown that increasing glucose levels to 18 and 15.5 mM, respectively, increases fibroblast proliferation, whereas further increases lead to an inhibition of proliferation [21,22]. Blood from uncontrolled diabetes commonly exhibits glucose amounts between 20 and 30 mM [23]. The amount of glucose concentration used in this study was 25 mM in a high glucose medium. Thus it is considered that PDL cell proliferation may be inhibited in patients with uncontrolled diabetes.

Other studies have shown the effects of high glucose concentration amounts in inhibiting the proliferation of proximal tubule cells, fetal mesangial cells and umbilical endothelial cells [24,25].

The reason for the reduced proliferation rate in a high glucose medium or diabetic condition has been explained in detail. Hehenberger and Hansson [22] showed that a glucose amount of 15.5 mM and above inhibited fibroblast proliferation and induced resistance of growth factor such as IGF-I and EGF. They [26] observed that conditioned media, derived from a Type II DM wound fibroblast culture, inhibited normal fibroblast proliferation. This proliferation was more inhibited as the ratio of the media from the diabetic wound fibroblast culture to media from normal fibroblast became larger. They stated that due to the amount of D-glucose in the media, the L-lactate levels increased in all cell types and that L-lactate production may play a role in the proliferation of fibroblast *in vitro* [26].

Several authors confirmed reduced growth rates for diabetic skin fibroblasts versus age-matched control fibroblasts [27,28]. Chronic non-diabetic wound fibroblasts had a similar proliferation rate as diabetic non-lesional fibroblasts but they had a significantly higher proliferation rate than the chronic diabetic wound fibroblasts. It seems likely that diabetic wound fibroblasts are impaired as a result of cell aging in combination with, or induced by, diabetes (intrinsic aging) and the wound environment (extrinsic aging) [28].

Diabetic condition also can affect bone density and metabolism [29–32]. Since many researchers showed that figures regarding bone turnover and metabolism have reduced, we wanted to determine the effects of high glucose on the mineralization of PDL cells *in vitro*. Our results clearly demonstrated that high glucose inhibited the mineralization of PDL cells *in vitro*.

Several previous studies showed that PDL cells had high alkaline phosphatase activity compared to gingival fibroblasts and exhibited osteoblast markers such as osteocalcin release and mineralized matrix formations, when supplemented media containing ascorbic acid, β -glycerophosphate and/or dexamethasone were given [5,14,20]. Mineral-like nodules became microscopic in size by day 14 and the nodules grew and could be identified macroscopically by day 20, when supplemented media containing ascorbic acid, β -glycerophosphate, and dexamethasone were given [15,16]. Thus, osteoblast-like cells can be derived from PDL cells in supplemented media. Based on previous studies, we used a supplemented medium containing 50 μ g/ml of ascorbic acid, 10 mM of β -glycerophosphate and 100 nM of dexamethasone to determine the effect of glucose on the mineralization of PDL cell cultures. Our study showed that the control PDL cells initiated mineralized nodules about day 14. Larger and much more mineralized nodules were formed as time passed. On day 21, mineralized nodules could be identified in the control group by the naked eye. Thus, PDL cells in the control group responded to supplemented media in the same way as other studies showed. This suggests that the PDL cells in this study have a unique ability to develop mineralized nodules.

The PDL cells in a low glucose supplemented medium had a larger calcified area five to six times bigger compared to the PDL cells in a high glucose supplemented medium. The PDL cells in a high glucose medium had decreased nodule formation, macroscopically and microscopically, on day 21, suggesting that differentiation into osteoblast-like cells was inhibited. Balint et al. [33] also showed that high glucose inhibited

the differentiation and calcium uptake of the MC3T3-E1 cell in vitro. Taken together, it is considered that high glucose disturbs the differentiation and calcification of PDL cells, as well as osteoblasts, in a supplemented medium.

In conclusion, cellular activity such as proliferation and differentiation are essential events for the healing of wounds and regenerating periodontal tissues. The decreased cellular activity of PDL cells due to high glucose concentration amounts may compromise healing and periodontal regeneration in diabetic patients.

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