

Original

Osteogenic Effects of Glucose Concentration for Human Bone Marrow Stromal Cells after Stimulation with *Porphyromonas gingivalis* Lipopolysaccharide

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Abstract: Diabetes mellitus (DM) is a common disease occurring worldwide. Patients with DM are at an increased risk of losing their teeth compared to other individuals. DM increases the risk and severity of chronic inflammatory periodontal diseases, in which, bone resorption is found to occur. Chronic inflammation contributes to the development of DM and DM-associated complications. Hyperglycemia is a hallmark of DM and may contribute to sustained inflammation by increasing the levels of proinflammatory cytokines. However, the mechanisms by which bone-related complications develop in DM after stimulation by the *Porphyromonas gingivalis* lipopolysaccharide (*P. gingivalis* LPS) remain unknown. Results of studies performed to understand the effect of high glucose concentrations on the functions of osteoblasts are contradictory because some have suggested a subsequent increase (although others have suggested a decrease) in the rate of the biomineralization process. Therefore, we evaluated the effect of high glucose levels on the biomineralization and inflammation markers in a human osteoblastic cell line, after stimulation with LPS from *P. gingivalis*. We treated cells with glucose at concentrations 5.5, 8.0, 12 and 24 mM after stimulation with *P. gingivalis* LPS (1.0 mg/ml) and determined its effects on cell proliferation, alkaline phosphatase (ALP) activity, osteocalcin (OCN) production, calcium deposition, inflammatory cytokines, and osteogenic cytokines. Results demonstrated that high concentrations of glucose increased cell proliferation, but the ALP activity decreased at concentrations of 12 and 24 mM. Additionally, OCN production and Calcium (Ca) deposition decreased at 24 mM. The levels of inflammatory cytokines (IL-1 β , IL-6 and IL-8) decreased at 8.0 and 12 mM. However, the amounts of Runx2 increased significantly in the presence of 12 mM glucose, but decreased beyond this concentration. High glucose concentration decreased hard tissue formation after stimulation by *P. gingivalis* LPS. Understanding the effect of glucose on osteoblast differentiation and calcium deposition might provide a better understanding of the development and prevention of periodontitis associated with diabetes.

Key words: Bone marrow cells, Hyperglycemia, *Porphyromonas gingivalis*

Introduction

Tooth loss, as a result of periodontitis, is a major dental health concern¹⁾. It is believed that inflammation of this periodontal tissue is strongly related to the occurrence of DM, one of the largest public health problems globally²⁾. Clinical studies on the complications of diabetes have implied that it may be a potential risk factor in the development of periodontitis^{3, 4)} and periodontal inflammation may negatively impact glycemic control⁵⁾.

The symptoms of periodontal diseases in individuals can be correlated with the severity of their diabetes. Periodontal disease is an inflammatory disease caused in part by the colonization of gingival and periodontal pockets by Gram-negative bacteria. Gingivitis can lead to periodontitis and subsequently to the loss of both periodontal ligaments and the alveolar bone^{6, 7)}. Diabetes is a major risk factor involved in the development of periodontal diseases. Individuals with either type 1 or type 2 diabetes experience exacerbated periodontitis compared to the non-diabetic subjects⁸⁻¹¹⁾. In general, people with diabetes exhibit in-

crease. The levels of proinflammatory cytokines such as tumor necrosis factor (TNF)- α and interleukin(IL)-6 have been found to increase in the serums of diabetics with periodontitis¹²⁾. However, controversies on the two-way interaction between periodontal clinical parameters and the onset and severity of diabetes still exist¹³⁾. A better understanding of the relationship between these two diseases is necessary before therapeutic interventions can be developed.

The viability and activation of periodontitis-related inflammatory signaling of human bone marrow mesenchymal cells (BMMCs) under the influence of hyperglycemia, presence of the *P. gingivalis* LPS and matrix glycation were investigated using a novel *in vitro* model. The results obtained from this study will enable clinicians to better understand the pathogenesis of periodontitis as well as its interplay with diabetes. The purpose of the present study was to determine the effect of prolonged elevated glucose concentrations on osteoblastic bone formation under influence of the *P. gingivalis* LPS.

Materials and Methods

Cell culture

BMMCs (PromoCell, Heidelberg, Deutschland) were seeded in T-75 flasks (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ, USA)

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and maintained in Eagle's minimal essential medium (EMEM; Nacalai Tesque Inc., Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS; Fraction V: Pierce Biotechnology, Rockford, IL, USA), penicillin (500 U/ml; Nacalai Tesque, Kyoto, Japan), streptomycin (500 µg/ml; Nacalai Tesque), and fungizone (1.25 µg/ml; Nacalai Tesque). Cells at passage stages 3–5 were seeded at a density of 5.0×10^4 cells/ml into 24-well or 96-well tissue culture plates (Becton Dickinson Labware) containing titanium discs. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂/95% air.

Cell proliferation assay

Cell proliferation was measured according to the manufacturer's protocol. Human BMMCs were seeded at a density of 4.0×10^4 cells/ml in normal culture medium (1 ml/well). After culturing for 24 hours to ensure cell adherence, the medium was replaced with one containing the *P. gingivalis* LPS (1.0 mg/ml; InvivoGen, San Diego, CA, USA) and incubated for 3, 6, or 72 hours. Fluorescence was recorded at 560/590 nm using a microplate reader. The difference between the two optical densities was defined as the proliferation value.

Cell differentiation at various glucose concentrations

After culturing the BMMCs for 7 days, the medium was removed and replaced with an osteogenic differentiation medium containing 10% FBS and osteogenic supplements, which were as follows: 10 mM β-glycerophosphate (Wako pure chemical, Osaka, Japan), 80 mg/ml ascorbic acid (Nacalai Tesque), 10 nM dexamethasone (Nacalai Tesque), and glucose (four different concentrations) with containing the *P. gingivalis* LPS (1.0 mg/ml). The glucose concentrations used during this study were chosen to reflect normal, postprandial and high values, similar to those observed in DM. Briefly, the normal, postprandial, and high glucose concentrations of 5.5 mM, 8.0 mM, 12 and 24 mM, respectively, were equivalent to 99 mg/dl, 144 mg/dl, 216 mg/dl and 432 mg/dl. The differentiation media were replaced every 2 days.

Alkaline phosphatase activity

After 2 weeks of osteogenic culturing with containing the *P. gingivalis* LPS (1.0 mg/ml), the cells were washed with PBS and lysed using 200 µl of 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA). The cell lysates were transferred to a microcentrifuge tube containing a 5 mm hardened steel ball. The tubes were agitated on a shaker (Mixer Mill Type MM 301; Retsch GmbH & Co., Haan, Germany) at 29 Hz for 20 seconds to homogenize the sample. The ALP activity was measured using the Alkaline Phosphatase Luminometric ELISA Kit (Sigma-Aldrich), according to the manufacturer's instructions. The reaction was terminated using 3 N NaOH to a final concentration of 0.5 N NaOH. The extent of p-nitrophenol production was measured by determining the absorbance at 405 nm using a 96-well microplate reader (SpectraMax M5; Molecular Devices, Sunnyvale, CA, USA). The DNA contents were measured using the DNA Assay Kit (Pico-Green dsDNA Assay Kit; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. In order to normalize the ALP activity, its levels were normalized to the amount of DNA in the cell lysates.

Osteocalcin analysis

The sandwich enzyme immunoassay used in this study was specific for osteocalcin and its levels could be measured directly in the culture supernatant after 3 weeks of osteogenic culturing with containing the *P. gingivalis* LPS (1.0 mg/ml). The osteocalcin levels in cell culture super-

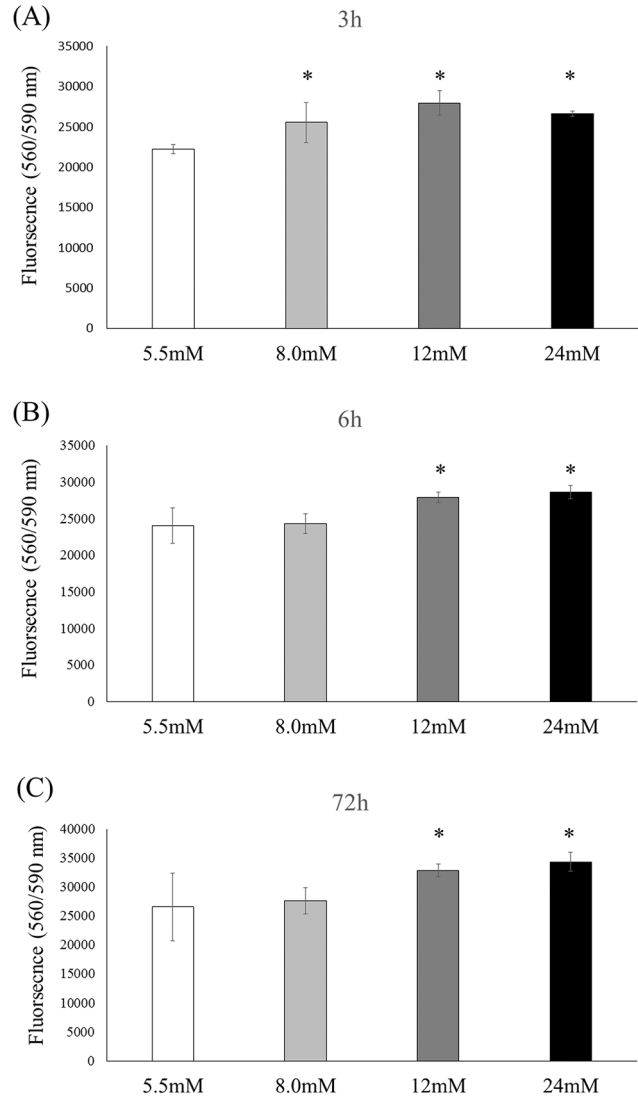


Figure 1. Cell proliferation. (A) after 3 hours of incubation, (B) after 6 hours of incubation, (C) after 72 hours of incubation in medium containing glucose at four concentrations (5.5, 8.0, 12 or 24 mM) measured by the CellTiter-Blue Cell Viability Assay. $P < 0.05$. *: vs 5.5 mM.

natants were measured according to the manufacturer's instructions (Osteocalcin ELISA Kit DS; DS Pharma Biomedical Co., Ltd., Osaka, Japan).

Extracellular matrix (ECM) mineralization

ECM mineralization by BMMCs was evaluated by alizarin red staining (Sigma-Aldrich). After 3 weeks of osteogenic culture with containing the *P. gingivalis* LPS (1.0 mg/ml), the cells were stained with alizarin red for 10 minutes at room temperature. The cell monolayers were washed with distilled water until the color was removed and images were acquired. Ca deposited in the ECM was measured after dissolution with 10% formic acid. The Ca levels were quantified using a Ca test kit (Calcium E-test Kit; Wako). After 3 weeks of osteogenic culturing, 1 ml of the Calcium E-Test chromogenic reagent and 2 ml buffer solution were added to 50 ml of the collected medium. Absorbance of the reaction products was measured at 610 nm using a 96-well microplate reader.

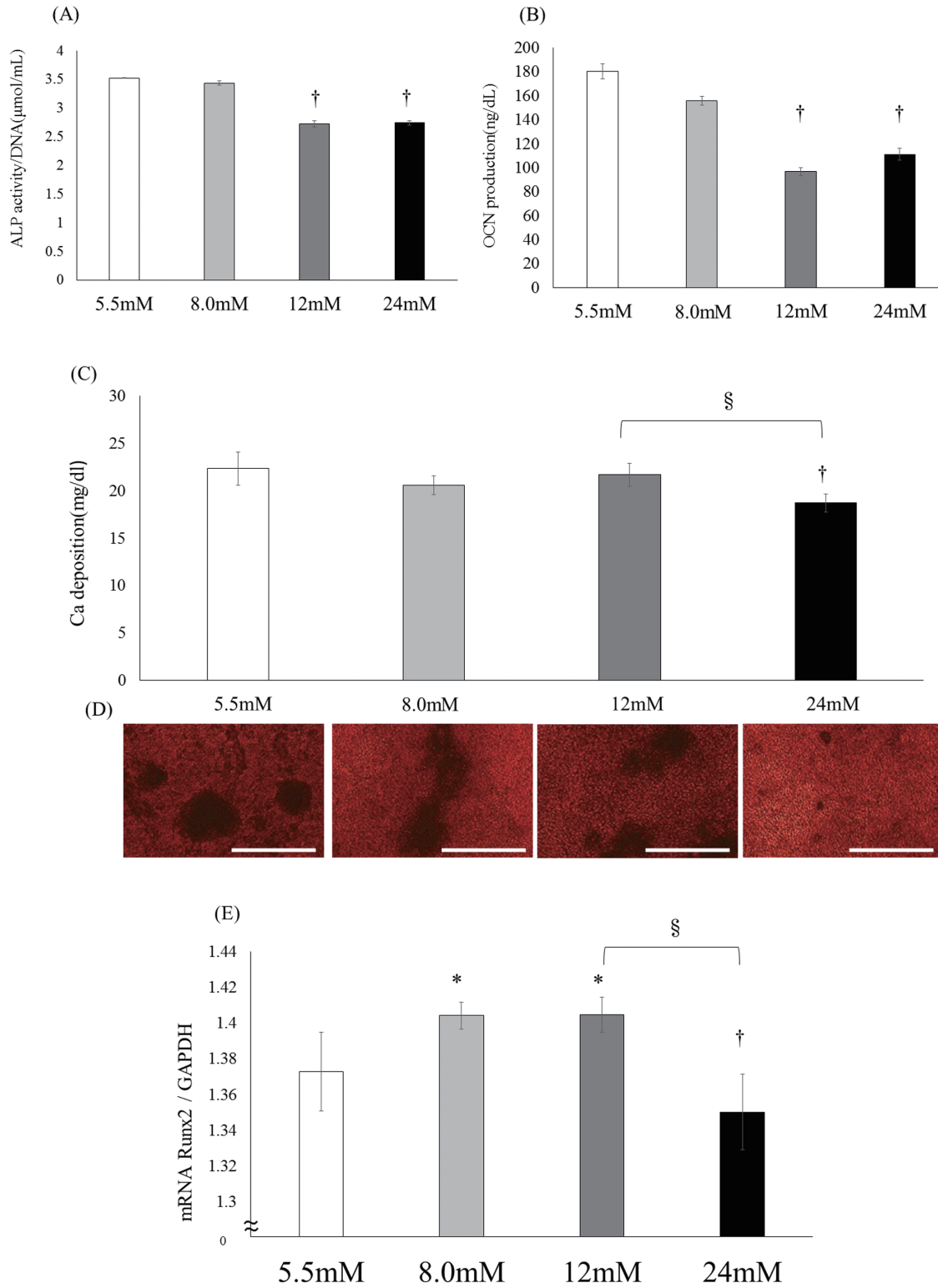


Figure 2. Effect of glucose concentration on osteogenesis. (A) ALP activity of BMMCs after 2 weeks of culture in osteogenic medium containing glucose at four concentrations (5.5, 8.0, 12 or 24 mM). (B) OCN production by BMMCs seeded after 3 weeks of culture in osteogenic medium containing glucose at four concentrations (5.5, 8.0, 12 or 24 mM). $P < 0.05$. [†]: vs 5.5 mM significantly low. (C) Ca deposition by BMMCs seeded after 3 weeks of culture in osteogenic medium containing glucose at four concentrations (5.5, 8.0, 12 or 24 mM). $P < 0.05$. [†]: vs 5.5 mM significantly low, [§]: vs 12 mM. (D) Ca deposition was stained by alizarin red. Scale bars are 100 μm . (E) Runx2 gene expression by BMMCs cultured after 3 weeks of culture in osteogenic medium containing glucose at four concentrations (5.5, 8.0, 12 or 24 mM). Data were obtained from real-time PCR analysis and are shown as means \pm SD expressed relative to GAPDH. $P < 0.05$. ^{*}: vs 5.5 mM significantly high, [†]: vs 5.5 mM significantly low, [§]: vs 12 mM.

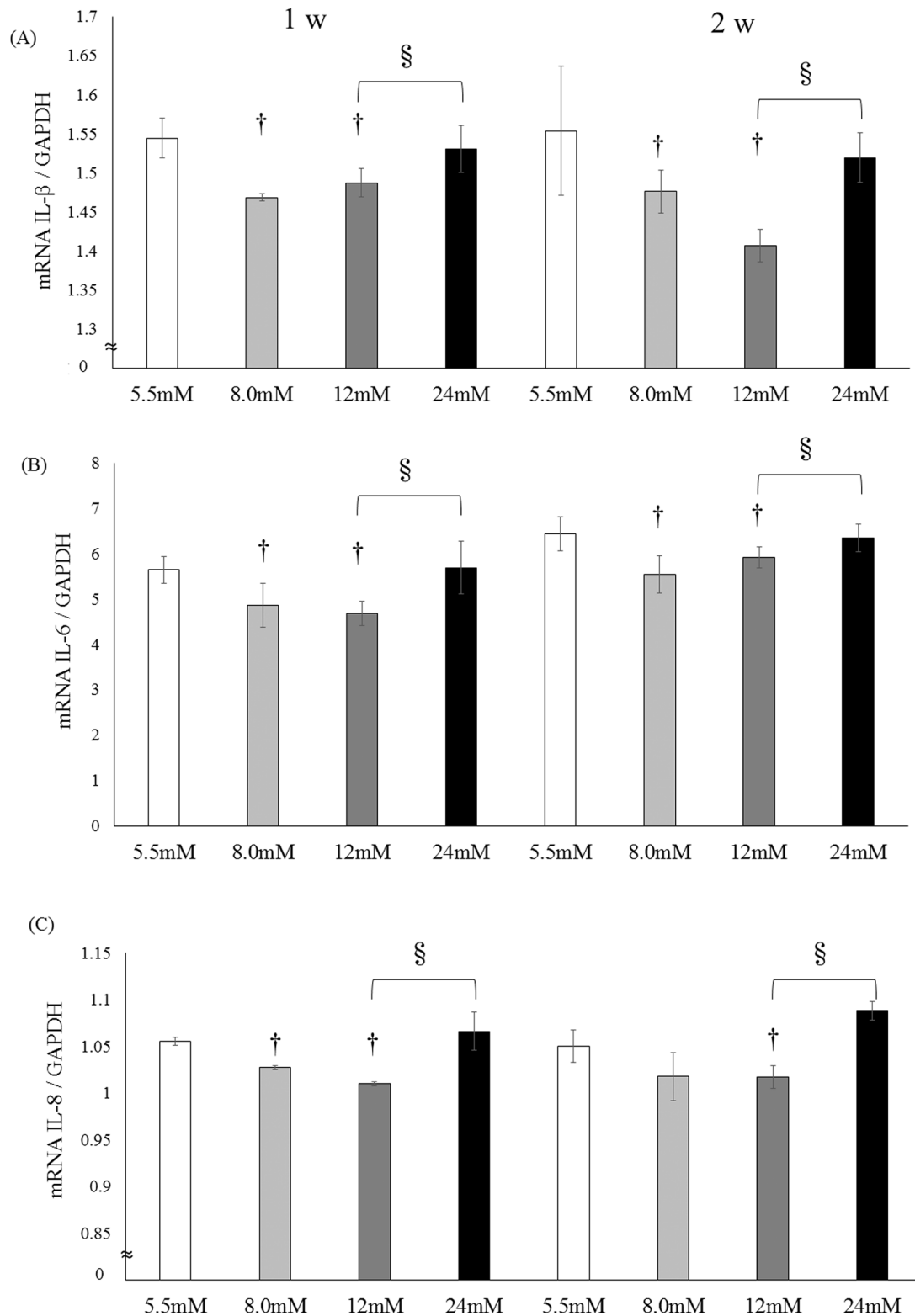


Figure 3. Inflammatory cytokine gene expression by BMMCs after 1 and 2 weeks of culture in osteogenic medium containing glucose at four concentrations (5.5, 8.0, 12 or 24 mM). (A) IL-1 β , (B) IL-6, (C) IL-8. Data were obtained from real-time PCR analysis and are shown as means \pm SD expressed relative to GAPDH. $P < 0.05$. *: vs 5.5 mM significantly high, †: vs 5.5 mM significantly low, §: vs 12 mM.

(Molecular Devices). The concentrations of Ca ions were calculated based on the absorbance values relative to a standard curve.

Gene expression

Gene expression was evaluated using a real-time reverse-transcription polymerase chain reaction (PCR) assay (TaqMan[®], Applied Biosystems, Thermo Fisher Scientific, Waltham, MA.). BMMCs were seeded at a density of 4.0×10^4 cells/ml in a normal culture medium (1 ml/well). After culturing for 7 days to enable cell adherence, the medium was replaced with the osteogenic differentiation medium containing glucose (5.5, 8.0, 12 or 24 mM) with containing the *P. gingivalis* LPS (1.0 mg/ml) and the cells were cultured further for either 1 or 3 weeks. The total RNA was isolated using an RNA extraction kit (RNeasy Mini Kit, Qiagen, Venlo, the Netherlands). RNA (10 ml) from each sample was reverse transcribed into a complementary DNA using a kit (PrimeScript[®] Reagent Kit, Takara Bio, Otsu, Shiga, Japan). The gene expressions for Runx2, IL-1 β , IL-6 and IL-8 (Taqman[®] Gene Expression Assay: Runx2, Rn01512298_m1; IL-1 β , Rn00580432_m1; IL-6, Rn01410330_m1; IL-8, Rn00174103_m1) were quantified using PCR (StepOnePlus Real-Time PCR System, Applied Biosystems, Thermo Fisher Scientific). The reactive gene expression rate for each group was calculated using the $\Delta\Delta C_t$ method, assuming that of the negative control group.

Statistical analysis

The data were analyzed using the SPSS 19.0 software (SPSS IBM, Chicago, IL, USA). All experiments were performed in triplicates. All data are expressed as the means \pm standard deviations (SD). In all the analyses, statistical significance was determined using the one-way analysis of variance (ANOVA) followed by a Fisher's least significant difference test. Values of $P < 0.05$ were considered as significant.

Results

Cell proliferation

Cell proliferation on the disks subjected to treatment with the *P. gingivalis* LPS after 3, 6 and 72 hours of culturing at four concentrations of glucose was assessed (Fig. 1). High glucose concentration promoted cell proliferation after culturing at all time.

Production of osteogenic proteins

ALP activities were determined at the four concentrations of glucose with the *P. gingivalis* LPS in osteogenic medium at 1 and 2 weeks of culturing. No difference was observed in the systems with glucose after culturing for 1 week. However, ALP activity had decreased significantly after 2 weeks in systems containing at 12 and 24 mM of glucose (Fig. 2A). The OCN production at these concentrations had decreased significantly after 3 weeks (Fig. 2B).

ECM mineralization

Ca deposition at 24 mM was significantly lower than that observed at other concentrations of glucose after 3 weeks (Fig. 2C).

ECM mineralization was assessed by alizarin red staining at 3 weeks of osteogenic culture (Fig. 2D). Additionally, the appearance of mineralization deposits differed according to the glucose concentrations.

The expression levels of cytokine genes of Runx2 was assessed by real-time PCR. The gene expression for Runx2 (Fig. 2E) had increased at 8.0 and 12 mM glucose concentrations, but decreased at 24 mM after 3 weeks.

Osteogenic and inflammatory cytokine expression

The expression levels of cytokine genes, including those for IL-1 β , IL-6 and IL-8 were assessed by real-time PCR. The gene expression for IL-1 β (Fig. 3A), IL-6 (Fig. 3B) and IL-8 (Fig. 3C) had decreased at 8.0 and 12 mM, but increased at 24 mM glucose concentrations after 1 and 2 weeks.

Discussion

Recent insights into the progression of periodontal disease have suggested that insufficient glycemic control is the most consistent risk factor associated with the severity and extent of a periodontal infection¹⁴. The current study examines the effects of chronic hyperglycemia on BMMCs derived from diabetic mice when stimulated with the *P. gingivalis* LPS, a common pathogenic by-product implicated in the pathogenesis of periodontal disease. It is known that hyperglycemia can alter the homeostasis of multiple body systems, including that of gene expression¹⁵⁻²⁰, but the mode of action by which diabetic hyperglycemia contributes to dysregulated innate immune responses, which subsequently contribute to the development of periodontal infection is not yet fully understood.

DM and periodontal disease occur as a degenerative cycle; a decline in the gingival health adversely affects metabolic glucose control, which is extremely important for maintaining good health of a DM patient²¹. In this study, a high concentration of glucose promoted the proliferation of BMMCs at an early stage after stimulation with the *P. gingivalis* LPS. However, abruptly elevated oxidative stress under high glucose concentration might increase the cytotoxicity in BMMCs, consequently reducing their viability. The *P. gingivalis* LPS is known to decrease biomineralization in osteoblasts²². However, although majority of our results involving high glucose concentrations indicated effects contrary to these, results of the study on the specific activity of ALP were unexpected. The effects of both LPS and high glucose treatments are similar, wherein the specific activity of the enzyme is diminished. Our data coincides with the study conducted by Zhen²³, although they are contrary to those from the study by Gopalakrishnan *et al.*²⁴, in which, high glucose levels had increased the activity of ALP. Our results coincide with the report by Shjoi *et al.*²⁵, who identified that the LPS demonstrated an inhibitory effect on the activity of ALP, but are contrary to those of Mo *et al.*²⁶, in which, the ALP activity had increased due to the LPS.

High concentrations of glucose inhibited the intracellular signal transduction in the osteoblast cells²⁷. Hence, ALP activity and OCN production was inhibited at glucose concentrations of 24 mM. However, our past study had suggested that high glucose concentration promoted OCN production because it acts on pancreatic β cells prompting the secretion of insulin, and a sensor for detecting glucose is present on the osteoblast cells.

High glucose condition is perceived due to the glucose ingested through GLUT1. OCN production is increased by high concentration of glucose. High OCN production had increased the Ca deposition²⁸. In this study, stimulation by the *P. gingivalis* LPS inhibited the OCN production. Moreover, Ca deposition was decreased at high concentrations of glucose.

The small size of nodules and perturbation of calcium deposition imply that the high glucose concentrations alter osteoblastic bone formation, which increase the volume of immature mineralization similar to that of calcium deposition evoked by the presence of LPS²⁹⁻³². Therefore, the only condition of high glucose concentration or stimulation by the *P. gingivalis* LPS is increased mineralization, but in both

conditions, the mineralization had decreased.

Runx2 promotes the uptake of glucose into the osteoblasts, and further acts to produce OCN. However, it is considered that the expression of Runx2 was reduced because glucose uptake into cells had decreased in a high glucose state. Our results coincide with those reported by Zhen *et al.*, in which, it was observed that higher glucose concentration (44 mM) had reduced mineralization³³⁾, but differ from those of the study by Li *et al.*³⁴⁾, Botolin and McCabe³⁵⁾, in which, they had determined that a high glucose increased mineralization. Although, contrary to our results, other reports that tested the effects of glucose at concentrations (15 and 24 mM) similar to the ones used by us observed a diminution in the mineralization process^{36, 37)}. Alternatively, Weil *et al.*³⁸⁾ determined that high glucose concentrations do not affect the production or proliferation of the human mesenchymal stem cell growth factor. Therefore, the contradictory effects of hyperglycemia on the biomineralization process observed in different studies may be due to heterogeneity of the cell source, differences in the experimental conditions and different concentrations of glucose tested.

In the previous study, we observed that an increase in mineralization was triggered only by high glucose concentrations (24 mM) in the BMMCs. But the biomineralization process seems to be affected by high glucose levels because they diminish the quality of minerals and modify the expressions of genes involved in the biomineralization and inflammatory processes.

Levels of the inflammatory cytokine were decreased at 8.0 and 12 mM glucose concentrations. Intake of glucose at concentrations as high as 12 mM was observed in the BMMCs and promoted the cell activity, but the oxidative stress was abruptly elevated due to high concentrations (24 mM) of glucose, which may increase the cytotoxicity, consequently reducing the viability of cells. Thus, the inflammatory cytokine had increased at 24 mM.

In the present study, alteration in the amount of osteocalcin released into the culture medium was detected, using either control or high glucose treatment. A very early phenotype characterized by low levels of OCN and high ALP expression was observed in cultures treated with high concentrations of glucose.

This fact further suggests that cellular differentiation is inhibited in the presence of high concentrations of glucose and supports the hypothesis that glucose has a negative regulatory effect on osteoblast differentiation.

Based on the results of the present study, since a high glucose concentration inhibits osteoblast maturation and calcium deposition leading to the formation of bone nodules after stimulation by the *P. gingivalis* LPS, it is possible that better a glycemic control might improve osteoblastic function and, subsequently, Ca deposition into the bone. Improved osteoblastic mineralization with increased Ca deposition into the bone would lower Ca concentrations in the serum. Therefore, under the same conditions of matrix glycation or stimulation with the *P. gingivalis* LPS, the response by BMMCs was also delayed and relatively modest in the activation of the ligand–receptor interaction, inflammatory signaling and matrix synthesis. Studies have also demonstrated that the differentiation capability of BMMCs could be significantly suppressed under matrix glycation or hyperglycemia³⁹⁾, further highlighting the importance of inflammation and glycemic control in recovering the regenerative capability of BMMCs during periodontitis. In summary, the present study is the first to demonstrate that elevated glucose concentration inhibits osteoblastic Ca deposition. The *in vitro* bone nodule formation assay was carried out after stimulation by the *P. gingivalis* LPS to demonstrate that glucose concentrations similar to those observed in pa-

tients with poorly controlled diabetes inhibits Ca deposition significantly. Because there is a reciprocal correlation between osteoblast proliferation and maturation, we propose that, in the presence of elevated glucose concentrations, osteoblast proliferation is enhanced while the *in vitro* bone formation is significantly inhibited. The metabolic alterations in diabetes include hyperglycemia, metabolic acidosis, and insulin insufficiency. It is likely that these factors co-contribute to the development of diabetic bone disease. The present study demonstrates that glucose exhibits a profound inhibitory effect on osteoblastic Ca deposition after stimulation by the *P. gingivalis* LPS. Understanding the effect of glucose on osteoblast differentiation and Ca deposition might provide a better understanding of the development and prevention of periodontitis associated with diabetes.

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Conflict of Interest

The authors have declared that no COI exists.

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