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Original

Glucose Affects the Quality and Properties of Hard Tissue in Diabetes Mellitus Model

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Abstract: Patients with diabetes mellitus (DM) are at an increased risk of tooth loss compared to healthy individuals. Although studies human subjects suggest that diabetes control is affected by periodontitis, there is scarce mechanistic evidence supporting its biological plausibility. Therefore, using type 2 DM rat bone marrow mesenchymal stromal cells (BMMSCs) were incubated with four concentrations of glucose (5.5, 8.0, 12.0 or 24.0 mM), the effects of different glucose concentrations on BMMSCs stemness and osteogenesis were evaluated. High glucose concentrations decreased the fluorescence intensity of β-actin, STRO-1, CD73 and CD90. Moreover, cell proliferation decreased at high glucose concentrations. Alkaline phosphatase activity was decreased at 12.0 mM and 24.0 mM. In contrast, osteocalcin production and calcium deposition were considerably increased at 24.0 mM. Differences in the calcium/phosphate ratio associated with various glucose concentrations were similar to calcium deposition. The mRNA expression of *Runx2* and inflammatory cytokines increased with increasing glucose concentration. The RANKL/OPG ratio decreased at high glucose concentrations. A high glucose concentration increased hard tissue formation, but the quality and stemness of the mineralized tissue decreased. Thus, hard tissue had a high risk of bone resorption in the case of uncontrolled diabetes even if periodontal treatment stabilized state of periodontitis for a moment.

Key words: Bone marrow cells, Hyperglycemia, Stemness, Hard tissue formation

Introduction

Diabetes mellitus (DM) is a low-grade chronic inflammatory disease that is attributed to either the lack of insulin produced by the pancreas or the invalidity of the insulin generated[1]. On the basis of the International Diabetes Federation, close to 463 million adults aged 20-79 years are affected by diabetes. Moreover, the number of individual with diabetes will rise to 700 million by 2045[2]. People with diabetes are at a rising risk of complications, such as periodontitis, cardiovascular disease, diabetic retinopathy, diabetic nephropathy, and diabetic neuropathy. In type 1 diabetes mellitus (T1DM), the hyperglycemic situation because of an absence of insulin is related to various pathological changes including decreased bone mass and low bone mineral density. However, several studies associated with type 2 diabetes mellitus (T2DM) have presented conflicting results, with different reports exhibiting varying changes in bone mass levels such as a decrease, an increase, or no change[3]. The interplay between periodontal disease and diabetes has long been reported, with evidence showing complex interactions between these two distinct pathologies. Diabetes patients with difficulties in controlling serum glucose levels are more likely to suffer from periodontitis than those with sufficient diabetes control and those without diabetes, while periodontitis affects the management of diabetes control. Sustained chronic inflammatory responses have been widely implicated in the progression of T2DM[4]. Increased proinflammatory cytokine levels of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6 have been shown to promote multiple DM-associated processes such as pancreatic β-cell destruction and insulin resistance[5].

In this regard, uncontrolled T2DM is recognized as a factor of disordered bone metabolism and homeostasis, leading to it becoming a risk factor for the development or exacerbation of osteoporosis, fractures, periodontal disease, and orthopedic or dental implant failure[6].

Recently, it was revealed that the osteoclastic bone resorption that occurs during periodontitis is dependent on the receptor activator of NF-κB ligand (RANKL) produced by osteoblastic cells. Immune cells give inherent cues for RANKL activation that takes place during periodontal inflammation. The acquaintance accumulated and experimental tools established in the "osteoimmunology" have made essential contributions to understanding of periodontitis pathogenesis. Mutually, the research of periodontitis has provided discernment into the field. There is good evidence for elevated levels of IL-1β, IL-6 and RANKL/OPG ratios in patients with diabetes and periodontitis as compared to patients with periodontitis alone as well as a quantitative relationship between these cytokines and glycemic control[7]. This study discusses the molecular mechanisms underlying periodontal bone loss by focusing on osteoimmune interactions and RANKL.

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Materials and Methods

Cell culture

Rat bone marrow mesenchymal stem cells (BMMSCs) were isolated from the femoral tissue of 8 weeks old Goto-Kakizaki (GK) rats (Shimizu Laboratory Materials Co., Ltd., Kyoto, Japan), a model of T2DM. This study was approved by the Osaka Dental University Institutional Animal Care and Use Committee (Apporoval No. 2010004). Rats were

sacrificed using 4% isoflurane (Pfizer Inc., New York, NY, USA), and the bones were taken from their hind limbs. The proximal end of the femur and the distal end of the tibia were cut off. A 21-gauge syringe (TERUMO Inc., Tokyo, Japan) was inserted into each cavity of the bone marrow, and the bone marrow cells were flushed from the cavity with proliferation medium containing Eagle's minimal essential medium (Nacalai Tesque Inc., Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA), antibiotic-antimycotic mixed solution (Nacalai Tesque) containing 500 U/ml penicillin, 500 μg/ml streptomycin, and 1.25 μg/ml amphotericin B. The cells were then seeded in a 75 cm^2 flask in an incubator at 37°C with 5% CO_2/95% air. The medium was changed every three days. Passages 3-5 cells were used in all the experiments.

Preparation of different glucose concentrations medium

Four kinds of glucose concentration media were prepared according to the fasting blood glucose level. Four glucose concentrations were added to the proliferation medium to induce cell proliferation. Osteogenic differentiation medium containing 10% FBS, antibiotic-antimycotic solution, and 80 mg/ml L-ascorbic acid (Nacalai Tesque), 10 mM β-glycerophosphate 2-phosphate (Nacalai Tesque), 10 nM dexamethasone (Nacalai Tesque), and glucose (5.5, 8.0, 12.0 and 24.0 mM) were prepared to induce osteogenic differentiation. The glucose concentrations used in this study were chosen to reflect normal, postprandial, and high glucose values, similar to those seen in DM. In brief, the normal glucose concentration of 5.5 mM is equivalent to 99 mg/dl, while the postprandial concentration of 8.0 mM corresponds to 144 mg/dl, and the high glucose concentrations of 12.0 mM and 24.0 mM are approximately 216 and 432 mg/dl, respectively. The proliferation and differentiation medium was changed every 3 days.

Immunofluorescence staining

GK rat BMMSCs were seeded in 24-well plates (Falcon tissue culture plate; Corning Inc., NJ, USA) at 5×10^3 cells/ml proliferation medium for 1 day for cell adhesion, the medium was removed and replaced with proliferation medium with four different concentrations of glucose, and allowed to incubate for 6 days. The cells were fixed with 4% paraformaldehyde, washed with PBS, and permeabilized with 0.5% Triton X-100 in PBS. The membranes were blocked with 3% bovine serum albumin-phosphate buffer solution (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) diluted in PBS and incubated overnight at 4°C with diluted primary mouse antibodies against β-actin (GeneTex Inc., Irvine, CA, USA), STRO-1 (Novus Biologicals, LLC, Centennial, CO, USA), CD73 (Proteintech Group Inc., Rosemont, IL, USA) CD90 (Bioss Antibodies Inc, Woburn, MA, USA) and CD105 (Bioss Antibodies Inc.). Immunofluorescent staining was performed using anti-rabbit IgG (Alexa Fluor 488®; Thermo Fisher Scientific Inc., Waltham, MA, USA) and anti-mouse IgG (Alexa Fluor® 488; Cell Signaling Technology, Danvers, MA, USA). Nuclei were stained with DAPI (Dojindo Molecular Technologies Inc., Kumamoto, Japan). Images were captured using an HS All-in-one Fluorescence Microscope (KEYENCE BZ-9000 version, KEYENCE CORPORATION, Osaka, Japan).

Transforming growth factor-beta1(TGF-β1) ELISA

GK rat BMMSCs were seeded into 24-well plates at a density of 5×10^4 cells/ml and cultured to confluence in proliferation culture medium with four different glucose concentrations (5.5, 8.0, 12.0 and 24.0 mM).

After 1 week culture, the proliferation culture supernatant was collected and measured using the sandwich enzyme immunoassay. The expression of TGF-β1 was measured with TGF-β1 ELISA Kit (Invitrogen, Thermo Fisher Scientific Inc.) according to the manufacturer's protocol.

Cell proliferation assay

GK rat BMMSCs were seeded at a density of 2×10^4 cells/ml into 24-well plates according to the manufacturer's protocol. After culturing BMMSCs for 24 hours, the medium was removed and replaced with proliferation medium with four different concentrations of glucose (5.5, 8.0, 12.0 and 24.0 mM), and incubated for 1, 3, 6, 24, 48, and 72 hours. At each time point, 100 ml Cell Count Reagent SF (Nacalai Tesque) was added to each well. After incubation at 37°C for 1 hour, the solution was transferred to a microplate reader at 405/650 nm absorbance. Data were analyzed using Soft Max® Pro Microplate Data Acquisition and Analysis software (Molecular Devices LLC, San Jose, CA, USA).

Alkaline phosphatase activity

GK rat BMMSCs were seeded into 24-well plates at a density of 5×10^4 cells/ml and cultured to confluence in proliferation culture medium. The medium was removed and replaced with osteogenic differentiation medium with four different glucose concentrations (5.5, 8.0, 12.0 and 24.0 mM).

After 1 week of osteogenic culture, cells were washed with phosphate-buffered saline (PBS) and lysed with 300 μl of 0.2% Triton X-100 (Sigma-Aldrich Co. LLC), and the cell lysates were transferred to collection tubes containing 2 mm hardened steel balls. The tubes were agitated on a shaker (Mixer Mill Type MM 301; Retsch Gmbh & Co., Haan, Germany) at 30 Hz for 20 seconds to homogenize the sample. Alkaline phosphatase (ALP) activity was measured using one-step p-nitrophenyl phosphate (pNPP; Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. To normalize ALP activity, DNA was measured using the DNA Assay Kit (PicoGreen dsDNA Assay Kit; Invitrogen) according to the manufacturer's protocol. Data were analyzed using the Soft Max® Pro software.

Osteocalcin ELISA

Cell osteogenic culture supernatants were collected and measured directly using the sandwich enzyme immune assay method after two weeks of osteogenic culture. Osteocalcin (OCN) levels in the culture supernatant were quantified using an OCN detection kit (Rat Osteocalcin ELISA Kit DS; DS Pharma Biomedical Co., Ltd., Osaka, Japan), in accordance with the manufacturer's protocol.

Extracellular matrix (ECM) mineralization

After 2 weeks of osteogenic culture, the cells were washed with PBS, fixed with 70% ethanol, and stained with Alizarin Red (Sigma-Aldrich Co. LLC) for 5 min at 25°C. After two weeks of osteogenic culture, cells were harvested with 10% formic acid, and the cell lysates were transferred to 2 ml microcentrifuge tubes and homogenized using the same method described above. Extracellular matrix calcium (Ca) deposition was measured using a Ca test kit (Calcium E-test Kit; FUJIFILM Wako Pure Chemical Corp., Osaka, Japan). The absorbance of the reaction solution was measured at 610 nm using a 96-well microplate reader (Molecular Devices LLC), following the manufacturer's protocol.

The cells were lysed with 10% formic acid and the phosphate (P) deposition in the ECM was measured. P detection kit (malachite green phosphate detection kit; BioAssay Systems Inc, Hayward, CA, USA) was used to quantify P levels. The working reagent (20 μl) was added to 80 μl of the acid reagent, and the absorbance of the reaction product was

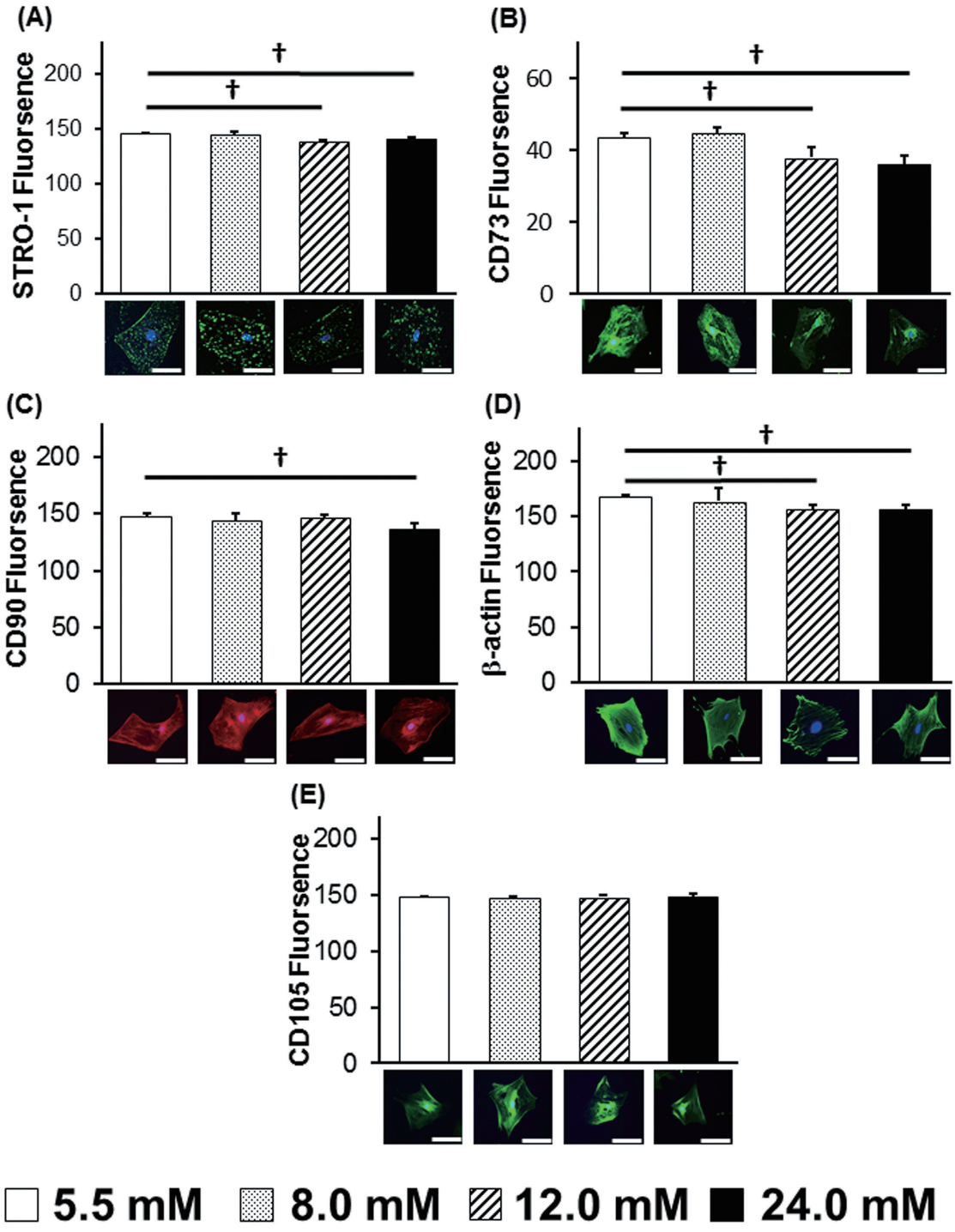

Figure 1. Fluorescence intensity and immunofluorescence staining of (A) STRO-1, (B) CD73, (C) CD90, (D) β-actin (E) CD105 after 1 week of incubation in medium containing glucose at four concentrations (5.5, 8.0, 12 or 24 mM). $P < 0.05$, *: vs 5.5 mM. Scale bars are 100 μm

measured at 620 nm using a 96-well microplate reader (Molecular Devices LLC). The concentration of each P ion was calculated from the absorbance value relative to a standard curve.

Gene Expression

Gene expression was determined using quantitative real-time polymerase chain reaction (RT-PCR) assay (TaqMan; Applied Biosystems, Thermo Fisher Scientific Inc.). GK rat BMMSCs were seeded into 96-well plates at a density of 5×10^4 cells/ml with proliferation culture medium to confluence, the medium was replaced with osteogenic differentiation medium containing four kinds of glucose concentration, and cells were cultured for 1 week. Total RNA was lysed using lysis buffer and isolated using an RNeasy Mini Kit (Qiagen Inc, Venlo, Netherlands). Total RNA from each sample was reverse transcribed into double-stranded DNA using a PrimeScript Reagent Kit (Takara Bio Inc, Otsu, Shiga, Japan). Gene expression for runt-related transcription fac-

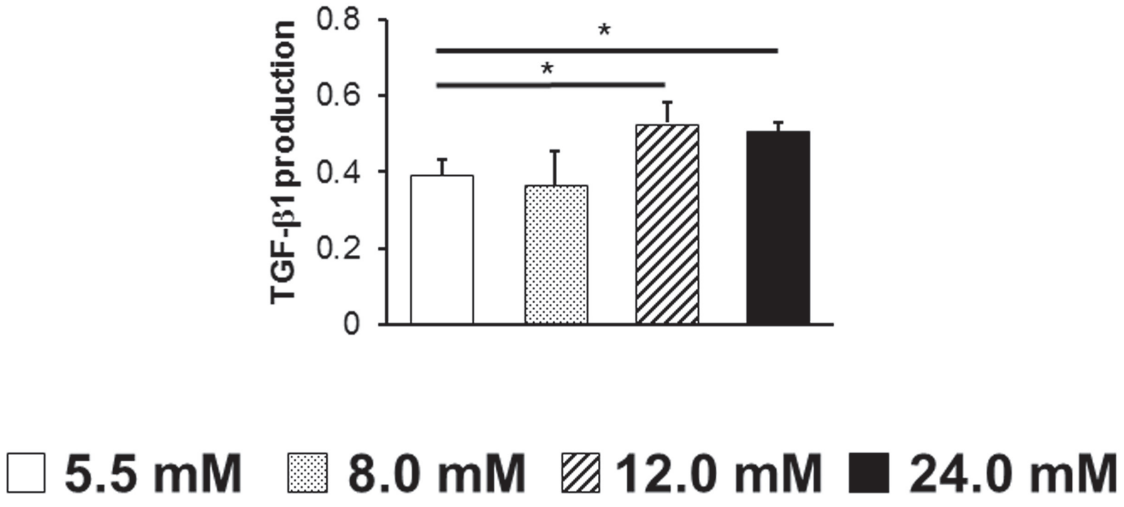

Figure 2. TGF-β1 gene expression after 2 weeks of culture in osteogenic medium containing glucose at four concentrations. Data were obtained from real-time PCR analysis and are shown as means ± SD expressed relative to GAPDH. $P < 0.05$, *: vs 5.5 mM

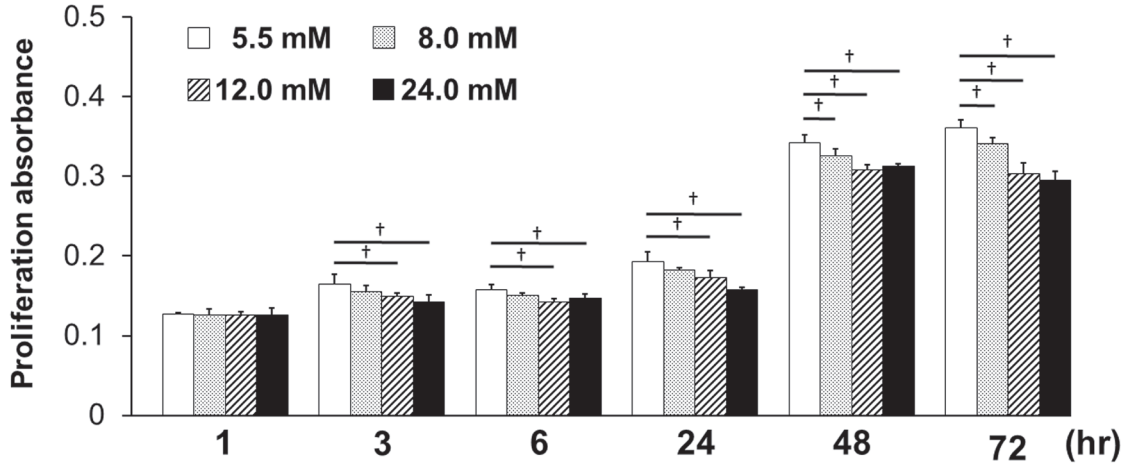

Figure 3. Cell proliferation after incubation in medium containing glucose at four concentrations measured by the Cell Count Reagent SF. $P < 0.05$, †: vs 5.5 mM significantly low

tor 2 (*runx2*) tumor necrosis factor-alpha (*tnf-α*), interleukin (*il*)-*1β*, *il-6*, receptor activator of nuclear factor kappa-B ligand (*rankl*) and osteoprotegerin (*opg*) (Taqman Gene Expression Assay, *runx2*: Rn01512298_m1, *tnf-α*: Rn01525859_g1, *il-1β*: Rn00580432_m1, *il-6*: Rn01410330_m1, *rankl*: Rn00589289_m1, *opg*: Rn00563499_m1) was performed by StepOnePlus Real-Time PCR System (Thermo Fisher Scientific Inc.). The reactive gene expression of each group was calculated using the ΔΔCt method at different time-points and normalized to GAPDH expression.

Statistical analysis

Data were analyzed using SPSS software, version 19.0 (International Business Machines Corporation, Armonk, NY, USA). All experiments were performed in triplicate. All data are presented as the mean ± standard deviation (SD). In all analyses, statistical significance was determined using one-way analysis of variance (Abnova Corporation, Taipei, Taiwan) followed by Fisher's least significant difference test. Statistical significance was set at $P < 0.05$.

Results

Immunofluorescence-based staining and TGF-β1 production

The fluorescence intensity of β-actin, STRO-1, and CD73 decreased at glucose concentrations of 12.0 mM and 24.0 mM, while that of CD90 decreased at 24.0 mM (Fig. 1A-D). However, the fluorescence intensity of CD105 did not changed at all glucose concentration (Fig. 1E). The TGF-β1 production of the 12.0 mM and 24.0 mM were significantly higher than that of 5.5mM group (Fig. 2).

Cell proliferation

GK BMMSC proliferation did not change significantly after 1 hour of incubation. After 3, 6 and 24 hours of incubation, the 12.0 mM and 24.0 mM groups were considerably decreased. After 48 and 72 hours of culture, the 8.0 mM, 12.0 mM and 24.0 mM groups were significantly lower than those in the 5.5 mM group (Fig. 3).

Osteogenic differentiation and mineralization

The activity of ALP in the 12.0 mM and 24.0 mM groups was significantly lower than that in the group of 5.5 mM group after 10 days of osteogenic culture (Fig. 4A). As the glucose concentration increased, the

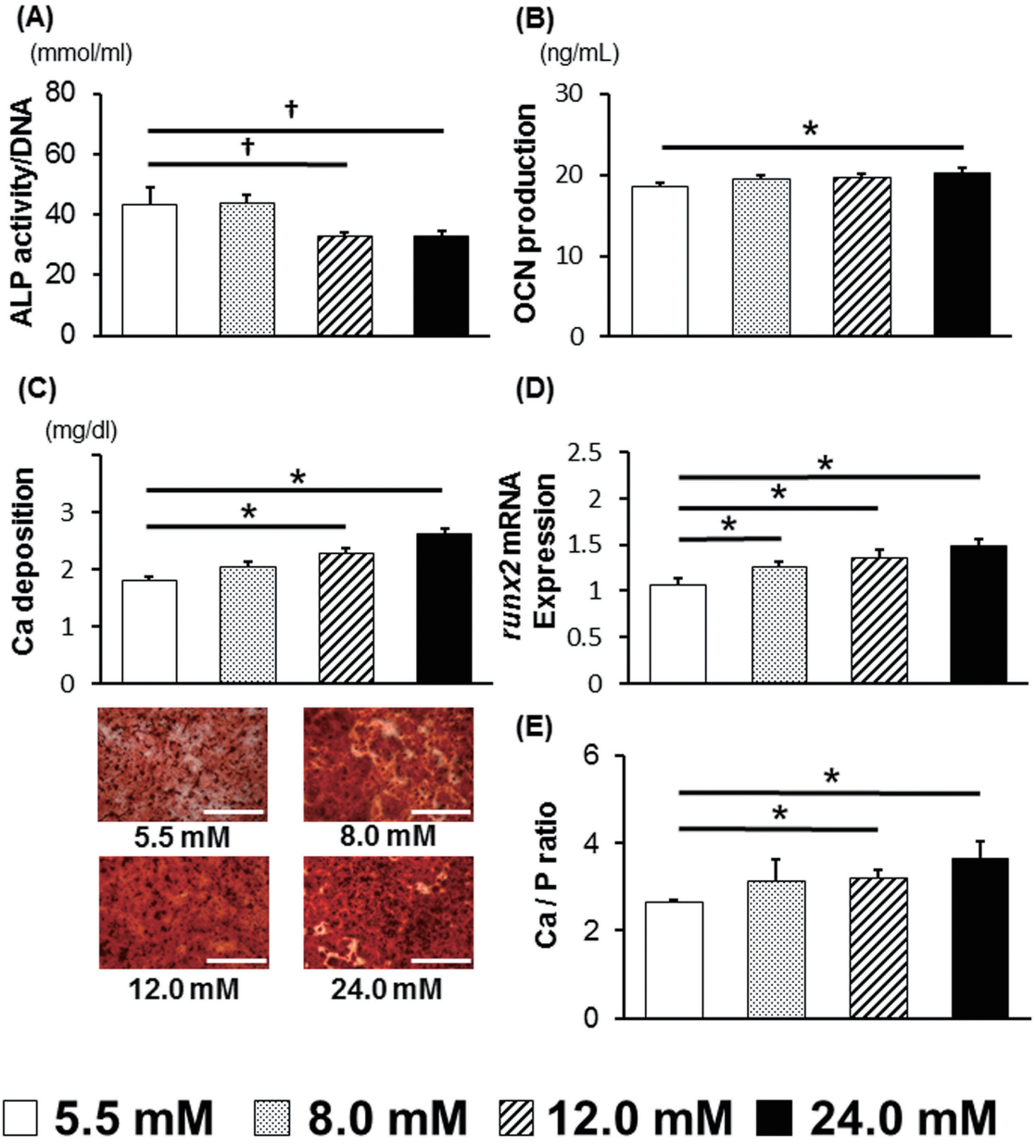

Figure 4. Effect of glucose concentration on osteogenesis. (A) ALP activity after 1 weeks of culture in osteogenic medium containing glucose at four concentrations. (B) OCN production after 2 weeks of culture in osteogenic medium containing glucose at four concentrations. (C) Ca deposition after 2 weeks of culture in osteogenic medium containing glucose at four concentrations. Ca deposition was stained by alizarin red. Scale bars are 100 μm. (D) Runx2 gene expression by BMMCs cultured after 2 weeks of culture in osteogenic medium containing glucose at four concentrations. Data were obtained from real-time PCR analysis and are shown as means ± SD expressed relative to GAPDH. (E) Ca/P ratio cultured after 2 weeks of culture in osteogenic medium containing glucose at four concentrations. $P < 0.05$, *: vs 5.5 mM significantly high, †: vs 5.5 mM significantly low

Ca deposition significantly increased. The results of Alizarin Red staining also showed the same phenomenon (Fig. 4C). OCN production significantly increased after 2 weeks of culture in the media containing 24.0 mM glucose (Fig. 4B). After 2 weeks of osteogenic culture. At 12.0 mM and 24.0 mM concentrations of glucose, the Ca/P ratio was significantly higher than that at the 5.5 mM group (Fig. 4E).

Expression of runx2 mRNA and Inflammatory cytokine

The expression of *runx2* mRNA in 8.0, 12.0 and 24.0mM was higher than that in 5.5 mM group as the glucose concentration increased after 1 week of osteogenic culture (Fig. 4D). The expression of inflammatory cytokine genes in the 24.0 mM glucose group, including *tnf-α*, *il-1β* and *il-6*, was significantly higher than those in 5.5 mM (Fig. 6A-C).

Expression of rankl and opg mRNA

After 2 weeks of osteogenic culture, the expression of *rankl* mRNA was increased at 12.0 mM and 24.0 mM (Fig. 5A). In contrast, the expression of *opg* mRNA was increased at 8.0 mM, 12.0 mM and 24.0 mM (Fig. 5B). Thus, the rankl/opg ratios of the 8.0 mM, 12.0 mM and 24.0 mM groups were increased significantly compared with that of the

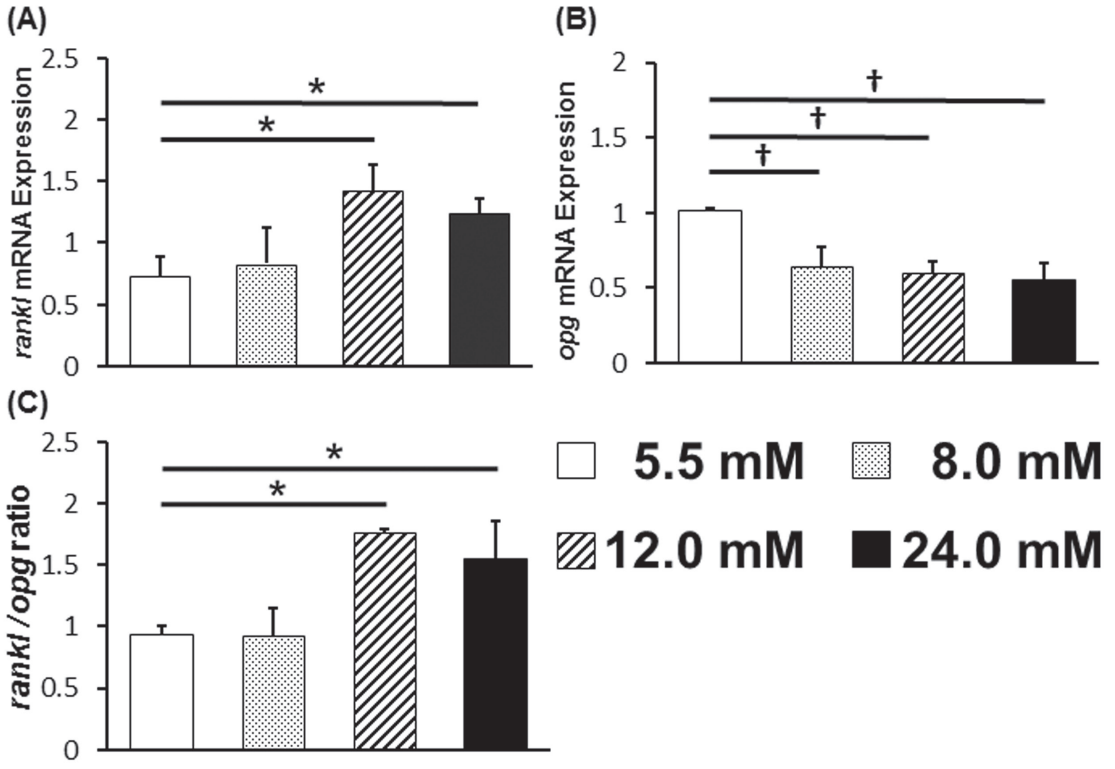

Figure 5. (A) RANKL gene expression by BMMCs cultured after 2 weeks of culture in osteogenic medium containing glucose at four concentrations. (B) OPG gene expression by BMMCs cultured after 2 weeks of culture in osteogenic medium containing glucose at four concentrations. Data were obtained from real-time PCR analysis and are shown as means ± SD expressed relative to GAPDH. (C) RANKL/OPG ratio. $P < 0.05$, *: vs 5.5 mM significantly high

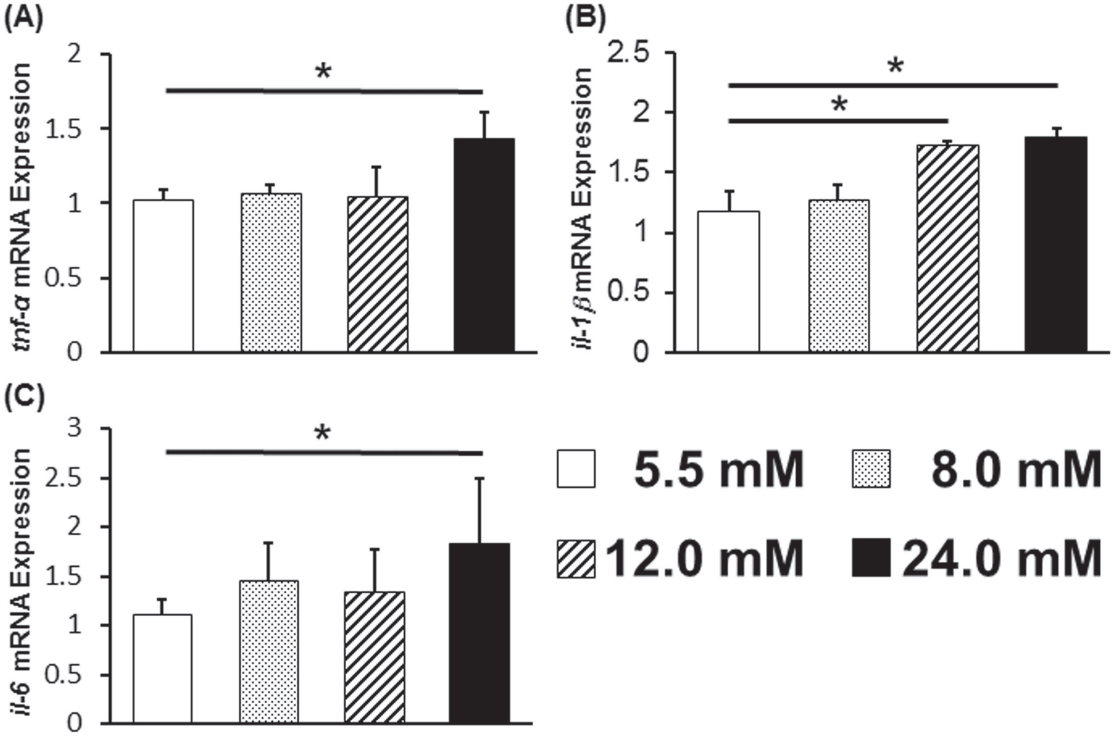

Figure 6. Inflammatory cytokine gene expression by BMMCs after 1 week of culture in osteogenic medium containing glucose at four concentrations. (A) TNF-α, (B) IL-1β, (C) IL-6. Data were obtained from real-time PCR analysis and are shown as means ± SD expressed relative to GAPDH. $P < 0.05$, *: vs 5.5 mM significantly high

5.5 mM group, especially of the 24.0 mM group (Fig. 5C).

Discussion

The correlation between periodontitis and DM has long been reported, with evidence showing complex interactions between these two distinct pathologies. Glucose concentrations ranging from 5.5 to 25 mM were selected to represent normal, postprandial, and high glucose conditions corresponding to those detected in diabetic patients[8]. Briefly, 5.5 mM represents normal glucose, 8.0 mM refers to postprandial, and 12.0, 24.0 mM are considered high glucose concentrations corresponding to the values seen in patients with decompensated diabetes.

Our results suggested that high glucose condition decreased cell stemness and cell proliferation of BMMSCs. A similar decrease in cell size, morphology, and cytoskeletal characteristics has been previously reported in heterogeneous rat BMMSCs populations following extensive *in vitro* culture expansion[9]. Prolonged expansion under persistent hyperglycemic conditions had limited impact on the proliferative capabilities of heterogeneous bone mesenchymal cell populations as a whole, actually stimulating proliferation between 50-300 population doublings[10]. Consequently, such findings are counterintuitive to the consensus that hyperglycemic conditions have negative influences on mesenchymal stem cell and osteoblast behavior[11]. Nevertheless, in line with high glucose conditions promoting BMMSCs, similar conclusions of hyperglycemia having positive or negligible effects on BMMSCs proliferation rates have also been reported[12].

T2DM develops as a consequence of pancreatic β-cell dysfunction together with insulin resistance in key insulin-regulated metabolic tissues (liver, skeletal muscle, and adipocytes). TGF-β superfamily members, including TGF-β, activin, and BMPs, control several developmental processes and have been implicated in pancreatic development and disease[13]. The molecule family is linked to osteoblastic proliferation, differentiation, activity, and collagen synthesis[14]. CD105 is a type-1 integral transmembrane glycoprotein and coreceptor for TGF-β ligands essential for fibrogenesis and angiogenesis[15]. We hypothesized that high-glucose concentration upregulation may decrease the fluorescence intensity of CD105. CD105 interacts with the TGF-β family by binding to TGF-β RI and RII at the cytoplasmic and extracellular domains, as well as serving as an auxiliary receptor to form TGF-β, β-glycan, the type III TGF-β receptor (TGF-β RIII). In such a quaternary configuration, the receptor complex is able to modulate TGF-β, SMAD1/5/8, and SMAD2/3 signaling. TGF-β and hypoxia upregulate CD105 expression, while CD105 (and β-glycans) regulate the suppression and enhancement of TGF-β related responses in certain cell types[16,17]. As a result, CD105 expression was elevated with increasing TGF-β expression to the same level of normal glucose concentration.

To prove the adverse effect of high glucose concentrations, mouse calvaria-derived cells were treated with normal glucose (5.5 mM) and high glucose (16.5 mM or 25.5 mM). High levels of glucose have been shown to significantly decrease proliferation, differentiation, and mineralization of osteoblasts due to hyperglycemia-induced ROS[18]. Further transcriptional analysis revealed that after incubation for 4 days, hyperglycemia reduced the expression of osteogenic markers such as alkaline phosphatase (ALP), while resorption-related markers such as matrix metalloproteinase-9 (MMP-9) and carbonic anhydrase II were found to be upregulated[19]. Noncanonical TGF-β signaling through the Erk MAPK pathway inhibits alkaline phosphatase expression, but promotes collagen synthesis[20].

Similarly, high glucose condition decreased ALP activity, but increased OCN in this study. OCN is the most abundant osteoblast-specific non-collagenous protein and a determinant of bone formation[21]. The hypothesis that bone-derived OCN regulates glucose metabolism originates from studies on OCN-null mice. In addition to impaired glucose metabolism, these mice also showed abnormal amounts of body fat accumulation and decreased peripheral insulin sensitivity. Both the gain-of-function and loss-of-function mouse models indicate that OCN plays a key role in the regulation of energy metabolism[22]. Thus, increasing OCN production helped BMMSCs adapt to the environment at high glucose concentrations. Moreover, Ca deposition was increased by high glucose concentration.

The cooperation of osteoblast differentiation and bone formation throughout life is maintained by a feed-forward regulation between uptake of glucose in osteoblasts and Runx2 cumulation. In addition, uptake of glucose by osteoblasts is necessary for whole-body glucose homeostasis. Independent of its regulation of bone formation, uptake of glucose in osteoblasts is necessary for another cardinal function of bone: the regulation of whole-body glucose metabolism. In fact, through its regulation of Runx2 accumulation, glucose favors the expression of the hormone OCN[23].

The effects of TGF-β1, β2, and β3 on osteoblastic cells are context-, time-, and dose-dependent, and differentially affect certain stages of osteoblast differentiation. Despite some conflicting findings, most experiments suggest that TGF-β promotes proliferation, early differentiation of osteoblast progenitor cells, and matrix production, while inhibiting later differentiation and matrix[24].

However, the quality of Ca deposition at high glucose concentrations was different from that at normal glucose concentrations. In high glucose concentrations, osteoblast proliferation is enhanced, whereas *in vitro* bone formation is significantly inhibited[25]. Not only bone density, bone quality also plays an important role in bone strength, and is closely related to the Ca/P ratio[26]. In this study, P deposition decreased at high glucose concentrations, and this phenomenon was associated with ALP activity. ALP is a glycoprotein that exists on cell membranes and can degrade inorganic phosphorus and alcohol to form phosphoric acid ester under alkaline conditions (pH 9-11). Indeed, high glucose concentration reduced ALP activity and P deposition and cause the Ca/P ratio to rise, so the balance between Ca and P is losed. We suggest that a high glucose concentration upregulates the volume of hard tissue formation, but downregulates the quality of hard tissue.

Since RANKL is the master regulator of osteoclastogenesis and plays an critical role in osteoclast-associated diseases, researchers in the field of periodontology have long explored the cellular source of RANKL and the mechanisms underlying RANKL induction during periodontitis. The levels of RANKL mRNA in the periodontal tissue and RANKL protein in the gingival crevicular fluid are associated with the severity of periodontitis in patients[27]. Among the cells that constitute the periodontal tissue, osteoblasts, osteocytes, periodontal ligament cells, and gingival epithelial cells have been shown to support osteoclastogenesis by producing RANKL *in vitro* co-culture systems[28].

OPG is a member of the TNF receptor superfamily and is also known as osteoclastogenesis inhibitory factor. Furthermore, studies have shown that OPG acts as a soluble decoy receptor of RAKNL, the main function of OPG being to antagonize the effects of RANKL and interrupt the crosstalk between osteoblasts and osteoclasts[29].

The major known signaling pathways of osteoblastic differentiation are RANKL/OPG[30], Wnt/β-catenin, 1,25-(OH)2-vitaminD3/VDR[31], and BMP-SMAD. In the RANKL/OPG rank system, cells secrete OPG, a protein involved in bone density regulation, to inhibit the differentiation of osteoclasts. The stromal cells of bone marrow, as well as osteo-

blasts, secrete RANKL, which promotes the differentiation of osteoclasts and bone resorption. Increasing RANKL/OPG ratio promoted the activity of osteoblasts by inhibiting osteoclastogenesis[32]. The increase in the ratio of RANKL/OPG in cultures stimulated with TNF-α is due to the increase in RANKL expression rather than the downregulation of OPG expression[33]. Furthermore, similar to published reports, a conditioned medium of osteocytes treated with TNF-α failed to support osteoclast formation, highlighting the importance of membrane-bound RANKL[34]. Evidence from clinical studies related to mediators of bone resorption such as RANKL/OPG ratio, as well as relevant animal models, strongly suggest that altered alveolar bone homeostasis is an important pathway of periodontal pathogenesis in diabetes, and there is evidence that this pathway may be important in both T1DM and T2DM[35].

In human mesenchymal stem cells, AGEs suppress the osteogenic differentiation of both cell types by increasing TGF-β expression[36]. Additionally, elevated fatty acid levels in patients with diabetes induce osteoclastogenesis through TNF-α[37]. The results of *in vitro* experiments with primary human and mouse osteoclast cells suggest that hyperglycemia may lead to increased formation[38]. Overall, the data support the concept that changes in the RANKL/OPG ratio and an inflammatory state in diabetes play an important role in alveolar bone resorption during periodontitis in individuals with diabetes. Thus, the volume of hard tissue formation upregulated but the quality of hard tissue downregulated and increasing mediators of bone resorption at high glucose concentration.

The dental management of patients with DM requires additional attention to their treatment planning and management, especially post-treatment. Proactive coordination of care with physicians to help ensure proper glycemic control is critical for long-term successful periodontal therapy. Patients with DM should be assessed regularly for periodontitis, and in those periodontally healthy, strict preventive measures should be instituted to prevent periodontal disease. If a patient with DM has periodontal disease, it should be treated definitively, reducing all periodontal pockets, establishing glycemic control, and controlling other complications managed in coordination with the patient's physician. Good glycemic control, as well as complete periodontal therapy and regular periodontal maintenance, will help the patient achieve a lifetime of good oral and general health.

Acknowledgments

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Conflict of Interest

The authors have declared that no COI exists.

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