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Osteoclasts in Bone Regeneration under Type 2 Diabetes Mellitus

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Abstract

Diabetes mellitus (DM) affects hundreds of million people worldwide and the impaired bone healing is an important DM-related complication. Understanding how DM affects the activities of osteoclasts and the underlying mechanisms is crucial to the development of effective approaches for accelerating bone healing in DM condition. To date, however, the influence of DM on osteoclasts remains obscure and controversial. In this study, we established a type 2 DM (T2DM) alveolar bone defect model, which closely simulates the pathogenesis of human T2DM, to explore the diabetic osteoclast activity during bone regeneration. We found that a high glucose concentration diminished the formation of osteoclasts, and the differentiation and function of osteoclasts from T2DM rats were suppressed. The degradation of matrix by osteoclasts was significantly reduced at a high glucose concentration. In vivo experiments further indicated that T2DM inhibited osteoclastogenesis and osteoclast activity, and delayed the degradation of matrix during the alveolar bone regeneration in T2DM rats. Our work clarifies the influence of T2DM on osteoclasts, and provides valuable insights for the design of novel scaffolding materials that target on osteoclasts for T2DM bone regeneration.

Graphical Abstract

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Conflict of Interest

The authors declare no conflict of Interest

Keywords

osteoclast; high glucose; bone regeneration; diabetes mellitus

1. Introduction

Diabetes mellitus (DM) affects hundreds of million people worldwide and the prevalence is increasing rapidly due to obesity and aging population [1]. Impaired bone healing is considered an important DM-related complication [2]. It was reported that the fracture healing time for DM patients was prolonged by 87% [3]. Our recent study also shows approximate 55% reduction of alveolar bone regeneration in type 2 DM (T2DM) condition [4]. Since bone healing and remodeling is regulated by the balance between osteoblasts (bone formation cells) and osteoclasts (bone resorption cells), understanding how T2DM affects the activities of osteoblasts and osteoclasts and the underlying mechanisms is crucial to the development of effective approaches for accelerating bone healing in T2DM condition.

While it has been well recognized that the capacity of mesenchymal stem cells (MSCs) differentiating into osteoblastic lineage is suppressed and the function of osteoblasts are impaired in a T2DM condition [5], the effect of T2DM on osteoclasts remains controversial. On the one hand, an in vitro study showed that the osteoclasts isolated from *Lepr^{db/db}* T2DM mice exhibited enhanced osteoclastic differentiation and higher bone resorption capacity [6]. Some animal experiments showed that the osteoclastic activity increased during the process of bone regeneration in T2DM, which contributed to delayed bone healing [7–9]. Graves and his colleagues demonstrated that T2DM aggravated periodontitis-induced bone loss and limited periodontal repair through enhanced osteoclastic resorption and decreased bone formation [10–12]. On the other hand, most of the human clinical investigations in recent years have demonstrated lower bone turnover in T2DM as indicated by decreased levels of bone formation and bone resorption, and the serum carboxy-terminal collagen crosslinks (CTX), a bone resorption marker, decreased in T2DM compared with the age-matched control group [13–17]. In vitro studies further indicated that high glucose concentration inhibited receptor activator of nuclear factor kappa-B ligand (RANKL)-mediated osteoclast differentiation and function [18–21]. In addition, impaired osteoclastogenesis was observed during the process of bone regeneration in T2DM animal models compared with the non-diabetic control group [22]. More confusingly, unchanged osteoclastic activity was also reported for both human and animal investigations in T2DM condition [23, 24]. Those conflicting results reflect the complex of the osteoclast level in T2DM, which is affected by

many factors, such as animal model selection, duration of T2DM, metabolic status, and medication used at the time of the measurements [25].

Osteoclasts, derived from the monocyte/macrophage haematopoietic lineage, play an essential role in bone healing. The intricate coupling of osteoclasts and osteoblasts regulates bone regeneration. For example, increased osteoclast activity during fracture healing led to the premature resorption of cartilage, which was responsible for diminished bone formation [26]. In addition to bone resorption, osteoclasts also influence bone regeneration by participating in the degradation of bone grafting materials such as calcium phosphate, hydroxyapatite and some polymers [27, 28]. The degradation of bone grafting materials creates space for the infiltration and formation of new bone. The malfunction of osteoclasts can either accelerate the degradation of bone grafting materials, resulting in the undesired release of a large amount of degradation byproduct and the attenuation of the supporting function of the graft for bone regeneration, or delay the degradation of bone grafting materials, leading to delayed bone formation.

Considering the critical role of osteoclasts in bone regeneration and the controversial results of osteoclast in T2DM, we established a T2DM alveolar bone defect model to explore the diabetic osteoclast activity during bone regeneration in this study. To closely simulate the pathogenesis of human T2DM, we chose to use high fat diet in combination with a low dose injection of streptozotocin (STZ) to establish the T2DM rat model. In our previous study, we found that alveolar bone regeneration was significantly impaired in the T2DM rat, and the application of scaffolding biomaterials with controlled release of cytokines was imperative to improve diabetic bone regeneration [4]. Therefore, biomimetic nanofibrous gelatin microspheres were implanted in the alveolar bone defect to identify osteoclast activity during T2DM bone regeneration, which sheds light on the development of novel biomaterials for T2DM bone tissue engineering.

2. Materials and Methods

2.1 Establishment of a rat T2DM model

Six-week-old male Sprague Dawley (SD) rats (Charles River Laboratories, USA) were used in this study. A T2DM model was established by feeding the rats with high-fat diets (D12451, Research Diets, Inc, USA) for 6 weeks in combination with a low dose STZ (30 mg/kg, Sigma-Aldrich, USA) injection. Fasting glucose over 11.1 mmol/l and stabling for 2 weeks were diagnosed as T2DM. After the T2DM confirmation, the animals were used for the following in vitro osteoclasts isolation and the in vivo alveolar bone defect experiments. All animal protocols were approved by the University Committee on Use and Care of Animals of Texas A&M University College of Dentistry.

2.2 Isolation of bone marrow macrophages and differentiation of osteoclasts

To investigate the effect of T2DM on osteoclasts, bone marrow derived osteoclasts were isolated from both 14-week-old healthy SD and T2DM rats. Briefly, the femurs and tibias were carefully dissected and the marrow cavity was flushed with α - minimum essential medium (MEM, Gibco, USA). After lysis of red blood cells, the cells were filtered through a

40 μ m cell strainer and cultured in α -MEM supplemented with 10% fetal bovine serum (FBS, Gibco), 1% penicillin-streptomycin (Sigma-Aldrich), and 50 ng/ml recombinant rat macrophage colony-stimulating factor (M-CSF, Peprotech, USA) for 7 days to differentiate into osteoclast precursors-macrophages. The cells were collected for the following experiments.

To identify the influence of high glucose on osteoclast viability, differentiation and function, osteoclast precursors were cultured in α -MEM containing different concentrations of D-glucose (5.6, 15, 25 or 50 mM) supplemented with 10% FBS, 1% penicillin-streptomycin, 50 ng/ml M-CSF and 50 ng/ml recombinant rat sRANK ligand (RANKL, Peprotech) for 5 or 7 days to generate osteoclasts. The medium was changed every 2 days. The 5.6mM concentration represented the normal glucose level in vivo (3.9–7.1mM), 15mM reflected the glucose concentration in diabetes patients (>11.1mM). Since diabetes is a chronic metabolic disease, the influence of high glucose on osteoclast differentiation is accumulated over time in vivo. The higher glucose concentration (25 mM) than that in diabetic patients in the in vitro experiment design could partially compensate for the time accumulating effects of high blood glucose in vivo. In addition, the use of 50 mM glucose concentration was to detect the effect of glucose concentration gradient on osteoclast differentiation.

2.3 Osteoclasts viability assay

Osteoclast precursors were seeded in 96-well culture plates at a density of 5×10^3 cells/well and cultured in α -MEM with various concentrations of D-glucose (5.6, 15, 25 or 50 mM) for 5 days. The cell viability was detected using a cell counting kit-8 (CCK-8, Sigma Aldrich). Briefly, after aspirating the culture medium from the well, 100 μ l α -MEM and 10 μ l CCK-8 solution were added into each well and incubated at 37 °C for 1 hour. The osteoclast viability was examined by measuring the absorbance at 450 nm using a microplate reader.

2.4 Qualitative reverse transcription polymerase chain reaction (qRT-PCR)

Osteoclast precursors were seeded in 12-well culture plates at a density of 5×10^4 cells/well and cultured in α -MEM with various concentrations of D-glucose (5.6, 15, 25 or 50 mM) for 5 days. The expression of osteoclastic markers tartrate-resistant acid phosphatase (TRAP), Cathepsin K and matrix metalloproteinase-9 (MMP-9) were detected by qRT-PCR. Total RNA was isolated from the cells using Trizol® reagent (Invitrogen, USA) according to the manufacturer's instructions. Reverse transcription was performed using a GoScript™ Reverse Transcription System kit (Promega, USA). The synthesized cDNA was applied to qRT-PCR using GoTaq® RT-qPCR System kit (Promega) and CFX96 Real-Time System (Bio-Rad Laboratories, USA). The sequences of primers (Invitrogen) used for qRT-PCR were listed in Table 1. GAPDH was used as an internal control for data analysis. The relative expression levels of mRNA were calculated via 2^{-Ct} .

2.5 TRAP staining

Osteoclast precursors were seeded in 8-well slide chambers at a density of 1×10^4 cells/well for TRAP staining. After differentiation for 5 days, the cells were fixed in 4% paraformaldehyde at 4°C for 30 min and permeabilized in 0.3% Triton X-100 (Sigma-Aldrich) for 15 min. After washing with PBS, TRAP solution was added into the well and

incubated at 37 °C for 15 min. Nuclei were further counter-stained with hematoxylin for 3 min. Images were acquired using an Olympus virtual slide microscope (Olympus BX61VS, Japan). TRAP positive cells with multiple nuclei (>3) were identified as osteoclasts. The number of osteoclasts was counted using an Image-Pro plus 6.0 software (Media Cybernetics, USA).

2.6 Resorption pit assay

Dentin slices were cut into 3×3 mm squares and immersed in 75% ethanol overnight for sterilization. After washing with PBS, dentin slices were placed into a 96-well culture plate. Osteoclast precursors were seeded on the dentin slices at a density of 1×10^4 cells/slice for 7 days. The attached cells were removed from the dentin slices using an ultrasonic processor (QSonica, USA) in cold PBS. Following dehydration through a gradient ethanol series, the dentin slices were vacuum dried and coated with gold using a sputter coater (SPI-Module Sputter Coater Unit, Japan). Images were acquired through scanning electron microscopy (SEM, JEOL JSM-6010LA, Japan). The area of resorption pits was measured via an Image-Pro plus 6.0 software.

2.7 Fabrication of nanofibrous gelatin microspheres (NFG-MS) and electrospinning nanofibrous gelatin membrane (NFG-MB)

NFG-MS were synthesized as previously reported [4]. Briefly, gelatin solution (12% in water/ethanol (1:1)) was added into mineral oil to form a water-in-oil (W/O) emulsion. The mixture was poured into isopropanol/1,4-dioxane/ethanol (4/3/1) mixture to induce phase separation and solvent exchange. The obtained NFG-MS were crosslinked, washed with distilled water, sieved to obtain different size, and lyophilized. Electrospinning nanofibrous gelatin membrane was fabricated according to our previous study [29].

2.8 Degradation of NFG-MS and nanofibrous gelatin membrane by osteoclasts

To explore whether osteoclasts degrade gelatin and the influence of high glucose on gelatin degradation, osteoclast precursors were seeded on NFG-MS or electrospinning nanofibrous gelatin membrane in 96-well culture plates at a density of 2×10^4 for 7 days. Immunofluorescent staining of actin was performed to visualize osteoclastogenesis on the surface of nanofibrous gelatin membrane. The cell-membrane constructs were fixed in 4% paraformaldehyde at 4°C for 30 min and permeabilized in 0.3% Triton X-100 for 15min. Following blocking with 20% goat serum and 3% BSA for 1 h, the samples were stained with CF®633 Phalloidin (Biotium, USA) at room temperature for 2 h and counter-stained with Hoechst (Invitrogen). Images were acquired using a confocal laser scan microscope (TCS SP5, Leica, USA). Attached cells on the NFG-MS were removed through an ultrasonic processor in cold PBS for SEM observation of gelatin degradation.

2.9 Establishment of the mandibular alveolar bone defect model

SD rats were randomly divided into 2 groups: the healthy control group (n=30) and the T2DM group (n=30). Mandibular alveolar bone defects were created in age-match healthy and T2DM rats as previously reported [4]. After the rats were anesthetized, the furs surrounding the surgical area were removed and the skin was disinfected. A 1.5 cm incision

was performed along the inferior border of the mandible to expose the masseter muscle. Following dissecting the masseter muscle and locating the buccal plate, a No.4 round bur was used to initiate access to the buccal root, followed by a No.1/2 bur to complete the osteotomy to create a standard defect size of 3×2×1mm. Sterilized NFG-MS were loaded into the defect area, and the muscle and the skin over the surgical site were repositioned and sutured. After surgery, all the rats were injected with analgesia for 3 d and fed with soft food diets for 7 d. The rats were sacrificed on 3, 7, 14, 28 and 56 d after surgery. Samples were harvested for RT-PCR and histology assay.

2.10 Histology assays

The rat mandibles were harvested at designed time points and fixed in 4% paraformaldehyde at 4°C for 48 h. The samples were washed in PBS for 1 h and demineralized in 15% ethylenediaminetetraacetic acid at room temperature for 6 weeks. After dehydration through a gradient ethanol series and paraffin embedding, consecutive horizontal sections (4 µm) were obtained from the middle third of the mandibular alveolar defect. The sections were dewaxed and rehydrated for the following staining.

Hematoxylin & eosin (H&E) staining was performed for histologic observation. Sections were stained in hematoxylin solution for 3 min, differentiated in 1% acid alcohol for 10 sec, immersed in 0.5% ammonia water for 10s for bluing, and counterstained in eosin solution for 10 sec. After dehydration and clearing, the sections were mounted with Permount mounting media. The double staining of ALP and TRAP was performed to simultaneously examine the osteogenic and osteoclastic activity. Following TRAP staining, the sections were washed with PBS and incubated in nitro blue tetrazolium chloride (NBT, Roche, Switzerland), 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Roche), and TNM buffer (100 mM Tris, 100mM NaCl, 5mM MgCl₂, PH 9.5) at 37°C for 25 min for ALP staining. Masson staining was conducted using the Trichrome Stain Kit (Sigma-Aldrich) according to the manufacturer's instruction.

2.11 Isolation of total RNA from alveolar bone defect and RT-PCR

After the rats were sacrificed, the mandibles were harvested and immersed in cold diethylenetriamine (DEPC) treated PBS. Attached soft tissue was removed from the mandible. Next, alveolar bone defect region was dissected and frozen in liquid nitrogen. The collected tissue was ground and crushed in liquid nitrogen and transferred to Trizol reagent. Following centrifugation at 12,000 rpm/min at 4°C for 10 min, the tissue residue was discarded and the Trizol supernatant was used for the isolation of total RNA according to the manufacturer's instruction. The isolated RNA was synthesized into cDNA and used for qRT-PCR. The primer sequences were listed in Table 1. Total RNA isolated from healthy rats without alveolar bone defect was used as a control for data analysis.

2.12 Statistical Analysis

Data were analyzed with SPSS software (version 16.0; SPSS, USA). All results were expressed as mean ± standard deviation. The difference between two groups was compared by student's *t*-test. The significance level was set at $p < 0.05$.

3. Results

3.1 High glucose levels inhibit osteoclast viability and differentiation

Bone marrow derived osteoclasts were cultured in differentiation medium with various D-glucose concentrations (5.6, 15, 25 or 50 mM) to determine the effect of high glucose levels on the viability of osteoclasts. As shown in Figure 1A, the viability of osteoclasts isolated from both healthy and T2DM rats gradually decreased with the increase of glucose concentrations. At the same glucose level, there was no significant difference of the osteoclast viability between the healthy group and the T2DM group.

The influence of high glucose on osteoclast differentiation was examined by detecting the expression of mature osteoclast markers. Compared to the healthy group, the expressions of TRAP, Cathepsin K and MMP-9 in the T2DM group at normal glucose level (5.6 mM) were down-regulated by 37%, 25% and 32%, respectively, indicating impaired differentiation of the osteoclasts isolated from the T2DM rats (Figure 1 B–D). The expressions of TRAP, Cathepsin K and MMP-9 of osteoclasts derived from both the healthy and T2DM rats decreased as the concentration of glucose increased from 5.6 mM to 50 mM. For instance, the expression of TRAP in the healthy group decreased by 11%, 27% and 79% in 15 mM, 25 mM and 50 mM glucose, respectively, compared to that in 5.6 mM glucose.

3.2 High glucose levels inhibit osteoclast TRAP activity and resorption function

TRAP staining was performed to identify the effect of high glucose on osteoclastogenesis. Figure 2A shows the macrophages isolated from healthy and T2DM rats were both differentiated into TRAP positive multi-nucleated osteoclasts under the stimulation of RANKL and M-CSF. However, at normal glucose level (5.6 mM), the number of osteoclasts derived from the healthy rats was 1.6-fold higher than that of the T2DM rats, and the size of the osteoclasts derived from healthy rats was also significantly larger than that of T2DM rats (Figure 2 A–C), suggesting the inhibited differentiation of osteoclasts derived from T2DM. Similar to gene expression, there was no significant difference in the number and size of osteoclasts derived from healthy and T2DM rats at a high glucose level (50 mM).

Bone resorption assay was conducted on dentin slices to examine the function of osteoclasts at high glucose levels. Corresponding to the TRAP activity, the area of bone resorption pits induced by osteoclasts derived from healthy rats was 2.1-fold higher than that of T2DM rats, demonstrating the down-regulated resorption function of the osteoclasts derived from T2DM rats (Figure 2 A&D). In addition, high glucose environment resulted in 86% and 73% reduction in the area of bone resorption pits induced by osteoclasts derived from healthy and T2DM rats, respectively, suggesting high glucose levels significantly suppressed the resorption function of osteoclasts.

3.3 High glucose levels suppress matrix degradation by osteoclasts

To identify the effect of high glucose on the degradation of gelatin matrix by osteoclasts, osteoclast precursors were seeded on both NFG-MS and NFG-MB in differentiation medium for 7 days. As shown in Figure 3 A&C, significantly larger resorption pits, accounting for 23.5% of the total area of the gelatin microsphere, were formed at the normal glucose level

(5.6 mM) compared to only 2.6% at the high glucose level (50 mM). To visualize the degradation of NFG-MB by osteoclasts, the NFG-MB were conjugated with fluorescein isothiocyanate (FITC) and the actin of cells was label with phalloidin. As shown in Figure 3 B&C, at normal glucose level (5.6 mM), multi-nucleated osteoclasts adhered and stretched on the surface of the NFG-MB, and obvious resorption pits were observed at the corresponding locations of the osteoclasts, indicating the osteoclasts were degrading the gelatin matrix. Furthermore, after the treatment of high glucose (50 mM), the number of multi-nucleated osteoclasts was significantly reduced and the matrix degradation was barely observed, suggesting high glucose inhibited the matrix degradation by osteoclasts.

3.4 Osteoclasts in T2DM exhibit a decreased activity and impaired matrix absorption during bone regeneration

A T2DM rat model was established through high fat diets combined with a low dose injection of STZ to simulate the pathogenic process of T2DM in humans. A mandibular alveolar bone defect model was further created and implanted with NFG-MS to investigate osteoclast activity during bone regeneration. ALP and TRAP double staining was performed to identify osteogenic and osteoclast activities.

On the third day after surgery, the ALP expression and TRAP positive osteoclasts were not detected in the defect area of the control group or the T2DM group (Figure S1). H&E staining showed a number of inflammatory monocytes/macrophages penetrated into the defect region. Some monocytes/macrophages were observed gathering around the NFG-MS and degrading the microspheres through phagocytosis and enzymolysis. Additionally, surgical trauma stress led to dramatically augmented bone remodeling outside the bone defect area, evidenced by the extensive and robust expression of the ALP and TRAP positive osteoclasts both in the control group and the T2DM group. Specifically, the number of osteoclasts significantly increased at the edge of the defect area (Figure S1).

On the 7th day after surgery, the ALP expression in the control group was significantly increased at the edge of the defect area and numerous TRAP positive osteoclasts were detected inside the defect region (Figure 4). Interestingly, most of the osteoclasts were observed adhering on the surface of the NFG-MS, where the degradation of the NFG-MS was also found. H&E staining showed a large number of multi-nucleated giant cells instead of monocytes/macrophages gathering around the NFG-MS in the control group, and the locations of these multi-nucleated giant cells were the same with the TRAP positive osteoclasts, indicating monocytes/macrophages fused or differentiated into osteoclasts after 7 days. However, the expression of ALP was weak in the T2DM group. Moreover, the osteoclast activity in the T2DM group was restricted to the edge of the defect region, and the number of osteoclasts was only 22% of that in the control group. H&E staining showed monocytes/macrophages still predominated around the NFG-MS and few multi-nucleated giant cells were observed, suggesting monocytes/macrophages fusing or differentiating into osteoclasts was significantly inhibited in T2DM condition. In addition, bone remodeling induced by surgical trauma remained active outside the defect region both in the control group and the T2DM group, although the expression of ALP and the number of the TRAP positive osteoclasts in the T2DM group were lower than that in the control group.

On the 14th day after surgery, abundant expression of ALP and plentiful TRAP positive osteoclasts were identified inside the defect region in the control group (Figure 5). It was apparent to observe osteoclasts adhering on the surface of NFG-MS and degrading the gelatin matrix. In H&E staining, multi-nucleated giant cells adhering on the NFG-MS remained the same position to that of the TRAP positive osteoclasts and few monocytes/macrophages were observed. Masson staining suggested new bone formation were initiated at the edge of the defect, and the degradation of gelatin matrix by osteoclasts created extra space for new bone formation in the control group. Whereas, the expression of ALP and the number of osteoclast in the T2DM group was only 28% and 37% of those in the control group on day 14, respectively. The distributions of ALP expression and TRAP positive osteoclasts in the T2DM group were close to the margin of the defect area, which was different from the extensive and even distribution of those in the control group. The size of osteoclasts in the T2DM group was significantly smaller than that in the control group, which was consistent with the in vitro result. H&E staining exhibited the co-existing of monocytes/macrophages and multi-nucleated giant cells on the surface of NFG-MS in T2DM, indicating some monocytes/macrophages fused or differentiated into multi-nucleated giant cells that were identified as TRAP positive osteoclasts. New bone formation was not detected by Masson staining under T2DM condition on day 14. It is worth noting that significantly more NFG-MS was observed in the T2DM group than that in the control group, suggesting the inhibited degradation of gelatin matrix under T2DM condition. Besides, bone remodeling outside the defect area in the control group remained significantly more active than that in the T2DM group.

On the 28th day after surgery, most of the defect region in the control group was occupied by new formed bone and only a small amount of non-degraded gelatin matrix was detected inside the defect region. By contrast, new bone formation and the extensive degradation of gelatin matrix was just initiated in the T2DM group (Figure S2).

On the 56th day after surgery, the defect area in the control group was occupied entirely by new formed bone and the implanted gelatin matrix was completely degraded (Figure 6). However, the defect area in the T2DM group was still in the process of bone regeneration including the degradation of gelatin matrix by osteoclasts and the formation of new bone, which explained significantly more osteoclasts in the T2DM group than that in the control group.

Semi-quantitative results of the ALP and TRAP double staining showed that osteoclasts were barely detected in the defect area in both the control group and the T2DM group on day 3 (Figure 7A). The numbers of osteoclasts in the defect area of the control group rapidly increased after 3 days, continued to rise to the peak on day 14, and gradually decreased to the basal level on day 56. However, the osteoclast number in the defect area of the T2DM group increased much slower and was significantly less than that of the control group. After a slow increase from 3 to 14 days, the number of osteoclasts reached a plateau until 28 days and then declined steadily. Intriguingly, even 56 days after surgery, osteoclast activity in the defect area of the T2DM group did not recover to its basal level and was higher than that of the control group. This result indicated that bone regeneration in the T2DM group was still ongoing and the osteoclasts were prerequisite for gelatin matrix degradation. In contrast,

new bone formation had completed and bone remodeling was initiated in the control group. The trend of ALP activity with time in the defect area was the same between the control group and the T2DM group, and the osteogenesis was dramatically down-regulated under the T2DM condition (Figure 7B).

Semi-quantitative analysis of TRAP positive osteoclasts surrounding the bone defect area showed the basal level of osteoclasts before surgery in the control group was approximately 2.4 times higher than that of the T2DM group (Figure 7C). In the control group, the osteoclast number increased sharply within the first 7 days after surgical trauma, and gradually decreased and recovered to the basal level on the 28th day after surgery. Although the trend of the osteoclast activity outside the defect area in the T2DM group was the same to that in the control group, the number of osteoclasts in the T2DM group was significantly lower than that in the control group during the whole process of bone regeneration. In addition, the ALP activity surrounding the bone defect area exhibited similar tendency and significantly down-regulated ALP expression was detected in the T2DM group (Figure 7D). These results indicated the decreased bone turnover under T2DM condition.

In addition to the histological observation and analysis, the RT-PCR was further performed to identify the gene expression of osteoclast markers and osteogenic markers in the defect region during the process of bone regeneration. TRAP, Cathepsin K and MMP-9 are the best known lysosomal enzymes released by osteoclasts. Specifically, the cysteine protease Cathepsin K and gelatinase MMP-9 are involved in collagen degradation. Gelatin, a denatured biopolymer derived from collagen by hydrolysis, possesses essentially the same composition as collagen. Therefore, the expressions of TRAP, Cathepsin K and MMP-9 not only indicate the formation of mature osteoclasts but also reflect the capability of degrading gelatin matrix by osteoclasts. As shown in Figure 8 A–C, the expressions of mature osteoclasts markers TRAP, Cathepsin K and MMP-9 in the T2DM group were significantly lower than those in the control group on day 7, 14 and 28, which was consistent with the reduced TRAP positive osteoclasts numbers in the T2DM group, further suggesting the obviously impaired osteoclast differentiation and decreased gelatin matrix degradation under T2DM condition. Similar to the histological observation, relatively higher expressions of osteoclast markers were identified in the T2DM group on day 56 after surgery. Meanwhile, the expressions of RANKL (essential for the formation and maturation of osteoclasts) and osteoprotegerin (OPG, binding and neutralizing RANKL) were detected to identify their regulation effect on osteoclasts during bone regeneration. Although there was no significant difference between the control group and the T2DM group on the RANKL/OPG ratio, the expressions of both RANKL and OPG in the T2DM group were significantly lower than those in the control group, which might contributed to the down-regulated osteoclastogenesis. Furthermore, dramatically decreased expressions of osteogenic transcription factors Runx2 and Sp7 on day 7, 14, and 28 further demonstrated the inhibited capacity of bone formation under T2DM condition, which was in line with the ALP staining result.

4. Discussion

Improving bone regeneration under DM condition has become an imperative problem because of the rapidly increasing incidence of diabetes. Although the mechanism of delayed bone healing under diabetic condition was extensively explored, most of those investigations mainly focused on the adverse impacts of diabetes on osteoblastic lineage cells. The influence of diabetes on osteoclasts, however, remains obscure and controversial. Osteoclasts play an essential role in bone tissue engineering, including participating in bone graft resorption and coupling bone formation. Given the impaired diabetic bone healing, the application of functional bone grafts to accelerate bone regeneration is indispensable, especially for tissue regeneration of large bone defect induced by periodontitis, trauma, or tumor. Malfunction of osteoclasts would offset the advantages of functional bone grafts [27] and even further deteriorate already impaired bone regeneration in diabetes [26]. In this study, we demonstrated that osteoclasts isolated from T2DM rats exhibited decreased osteoclastogenesis and resorption function compared to that from healthy rats. High glucose concentration inhibited the differentiation and function of osteoclasts derived from both healthy and T2DM rats (Figure 1–3). Moreover, gelatin matrix degradation by osteoclasts was significantly reduced at high glucose concentrations (Figure 3). In addition, in vivo alveolar bone healing experiment suggested that osteoclast differentiation and function was significantly down-regulated during the process of bone regeneration under T2DM condition (Figure 4–8, S1&S2), which further resulted in delayed degradation of the implanted bone graft.

T2DM is a low-grade chronic inflammatory disease, characterized by elevated expression of systemic inflammatory cytokines such as TNF- α , IL-1 β , and IL-6 [30]. In general, inflammation would be expected to lead to enhanced osteoclastogenesis and bone resorption [31]. However, rather than osteoporosis, bone mineral density (BMD) in T2DM patients is often normal or even increased by 5% to 10% compared with an age-matched control group [32]. In our study, high fat diets in combination with a single low dose injection of STZ was used to establish the T2DM rat model to simulate the pathogenesis of human T2DM. In comparison with healthy rats, T2DM rats exhibited a pronounced reduction of osteoclasts in physiological bone remodeling, which was in line with the decreased serum osteoclast marker CTX in T2DM patients reported in most of recent clinical investigations [13–17], indicating T2DM inhibited the formation and function of osteoclasts. We further demonstrated that the differentiation and function of osteoclasts derived from T2DM rats was suppressed compared to that from healthy rats, and high glucose concentration further diminished the formation of osteoclasts (Figure 1–3). On the contrary, most investigations using transgenic T2DM animal models - suggested increased osteoclast activity [7–9] and a study reported osteoclasts isolated from *Lepr^{db/db}* T2DM mice exhibited enhanced osteoclastogenesis [6]. It is worth noting that the spontaneous development of T2DM-like symptoms in transgenic T2DM animal models is a result of congenital deficiency of leptin receptor or leptin [33, 34], which is apparently different from the pathogenesis of human T2DM. Leptin participates in bone metabolism as an anabolic factor, stimulating osteoblast proliferation and differentiation while inhibiting osteoclastogenesis [35–37]. Therefore, enhanced osteoclastogenesis and decreased BMD in monogenic T2DM animal model such

as Lep^{ob/ob} mice, Lepr^{db/db} mice and Zucker Diabetic Fatty (ZDF) rats may be attributed to the congenital loss of leptin receptor or leptin. In view of the intrinsic increased osteoclast activity, it should be cautious to use transgenic T2DM animal models for investigation of bone regeneration in the future, especially with the application of absorbable bone grafts.

Coincided with the declined osteoclastogenesis during physiological bone remodeling in T2DM, our study further demonstrated significantly decreased osteoclast activity during bone regeneration in a prolonged inflammation condition (Figure 4–8), which is, however, in contrast with some other studies that suggested prolonged inflammation in T2DM impair bone repair through enhanced osteoclastic resorption and decreased bone formation [10, 11]. Besides the difference in T2DM animal models, the distinct microenvironment of bone regeneration may contribute to the contradictory results. For example, one study indicated T2DM increased osteoclastogenesis during the repair of alveolar bone defect induced by periodontitis in T2DM [11]. In that study, alveolar bone repair was performed in a bacterial environment that communicated with the oral cavity. On the contrary, another work from the same group showed T2DM decreased osteoclastogenesis in bacteria stimulated bone loss, in which equal amount of bacteria was inoculated at the scalp to induce calvaria inflammation [38]. Although both investigations were conducted under the stimulation of bacteria in the db/db T2DM mice, the results of osteoclastogenesis were opposite. A possible explanation is that T2DM increased the pathogenicity of oral microbiota, which lead to more severe periodontal inflammation and bone loss than microbiota from normal mice after transferring to germ-free mice [12], therefore, increased osteoclastogenesis in T2DM periodontitis bone repair. Unlike the two experiments mentioned above, bone repair in our experiment was carried out in a sterile environment and the prolonged inflammation was induced by the declined inflammation resolving ability instead of bacteria irritation. Additionally, the degree of inflammation in the microenvironment of bone regeneration in our study was apparently lower than that in periodontitis.

Nevertheless, the pathogenic mechanism of decreased osteoclastogenesis during bone regeneration in T2DM remains poorly understood. It is clear that high glucose inhibits RANKL induced osteoclastogenesis, and our study further demonstrated T2DM significantly diminished gene expression of RANKL during bone regeneration. In addition, smaller osteoclasts were formed in the T2DM group compared to the control group during bone regeneration. Predominance of osteoclasts instead of monocytes was observed 7 days after surgery in the control group (Figure 4). However, only a few smaller osteoclasts but a large number of monocytes were detected even 14 days after surgery in the T2DM group (Figure 5), which was in line with Toshiyuki's study that the fusion of monocytes into multinucleated osteoclasts were impaired during fracture healing in diabetic mice because of the down-regulation of DC-STAMP, a pivotal gene for osteoclast fusion a pivotal gene for osteoclast fusion [21]. Further studies are needed to clarify the specific mechanism of decreased osteoclastogenesis during bone regeneration in T2DM.

In consideration of the increasing application of functional bone grafts to improve diabetic bone regeneration, it is of great importance to understand osteoclast activity in T2DM. Our study, for the first time, demonstrated T2DM inhibited osteoclastogenesis and delayed matrix degradation during bone regeneration, which provides valuable clues to the design of

novel scaffolding materials for bone tissue engineering in T2DM. Based on this work, a moderate increase of osteoclast bioactivity at early stage might be an effective way to enhance bone healing in T2DM. Currently, we are working on incorporating several proteins or drugs in bone grafts, and utilizing the precisely controlled release of the proteins or drugs to regulate osteoclast bioactivity and bone regeneration in T2DM.

5. Conclusions

In this study, we established an alveolar bone defect T2DM rat model, which closely simulates the pathogenesis of human T2DM, and explored the diabetic osteoclast activity during bone regeneration. Our work indicated that the differentiation and function of osteoclasts derived from the T2DM rats were suppressed, and high glucose concentrations further diminished the formation of osteoclasts. At high glucose concentrations, the degradation of matrix by osteoclasts was significantly reduced. In vivo experiments further showed that the T2DM inhibited osteoclastogenesis and osteoclast activity, and delayed the degradation of matrix during bone regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Significance

Impaired bone healing is one of the diabetes mellitus (DM)-related complications. Understanding how DM affects osteoclast activity and scaffolding matrix degradation is pivotal to the development of effective approaches for accelerating bone healing in DM condition. Currently, the influences of DM on osteoclast activity and matrix degradation in bone defect areas, however, remain controversial and obscure. Herein, we established a type 2 DM (T2DM) alveolar bone defect model and our results show that T2DM inhibited osteoclastogenesis and osteoclast activity, and delayed the degradation of scaffolding matrix. Our work clarifies the influence of T2DM on osteoclasts and matrix degradation, and provides insights for the design of novel scaffolding materials that target on osteoclasts for T2DM bone regeneration.

Figure 1.

High glucose levels inhibit osteoclast viability and differentiation. (A) Viability of osteoclasts derived from healthy control rats (Con) and T2DM rats (DM) after cultured at various concentration of D-glucose (5.6, 15, 25, 50 mM). (B–D) Gene expressions of mature osteoclast markers (TRAP, Cathepsin K, MMP-9) at various concentration of D-glucose, ** $p < 0.01$.

Figure 2.

High glucose levels inhibit osteoclast TRAP activity and resorption function. (A) TRAP activity and resorption pits of osteoclasts derived from healthy control rats (Con) and T2DM rats (DM) after cultured at normal D-glucose level (5.6mM) or high D-glucose level (50mM). (B–C) Semi-quantification analysis of osteoclast number and size in TRAP staining. (D) Semi-quantification analysis of resorption pits area/total area of the dentin slides, ** $p < 0.01$.

Figure 3.

High glucose levels suppress gelatin matrix degradation by osteoclasts. (A) SEM images of the degradation of nanofibrous gelatin microspheres (NFG-MS); the yellow arrows indicate the resorption pits on NFG-MS induced by osteoclasts. (B) Confocal images of the degradation of nanofibrous gelatin membrane (NFG-MB) induced by osteoclasts; the white arrows indicate the multinucleated osteoclasts and the resorption pits on NFG-MB. (C) Semi-quantification analysis of resorption pits area/total area of NFG-MS and NFG-MB, ** $p < 0.01$.

Figure 4.

Suppressed osteoclastogenesis in T2DM on the 7th day after surgery. (A–D) ALP & TRAP double staining of the mandibular horizontal sections; the black dotted line marks the alveolar bone defect region. (E–H) High magnification of ALP & TRAP double staining and H&E staining inside the defect area; the black arrows in H&E staining indicate the multinucleated giant cells. (I–J) H&E staining of the alveolar bone defect area. (K–L) Semi-quantification analysis of osteoclast numbers and ALP positive area/region of interest (ROI) inside and outside the alveolar bone defect area, $**p < 0.01$.

Figure 5.

Inhibited osteoclast formation and gelatin microspheres degradation in T2DM on the 14th day after surgery. (A–D) ALP & TRAP double staining of the mandibular horizontal sections; the black dotted line marks the alveolar bone defect region. (E–H) High magnification of ALP & TRAP double staining and H&E staining inside the defect area; the black arrows in H&E staining indicate the multinucleated giant cells. (I–J) H&E staining of the alveolar bone defect area. (K–L) Masson trichrome staining of the alveolar bone defect

area. (M–N) Semi-quantification analysis of osteoclast numbers and ALP positive area/region of interest (ROI) inside and outside the alveolar bone defect area, ** $p < 0.01$.

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Figure 6.

Delayed gelatin microspheres degradation and bone regeneration in T2DM on the 56th day after surgery. (A–D) ALP & TRAP double staining of the mandibular horizontal sections; the black dotted line marks the alveolar bone defect region. (E–H) High magnification of ALP & TRAP double staining and H&E staining inside the defect area; the black arrows in H&E staining indicate the multinucleated giant cells. (I–J) H&E staining of the alveolar bone defect area. (K–L) Masson trichrome staining of the alveolar bone defect area; yellow arrows indicate the remaining undegraded gelatin microspheres (white). (M–N) Semi-

quantification analysis of osteoclast numbers and ALP positive area/region of interest (ROI) inside and outside the alveolar bone defect area, ** $p < 0.01$.

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Figure 7.

Decreased osteoclast formation and ALP activity during bone regeneration in T2DM accompanied with lower bone turnover. (A–D) Semi-quantification analysis of osteoclast numbers and ALP positive area/region of interest (ROI) inside and outside the alveolar bone defect area at different time points (3, 7, 14, 28, 56d) during bone regeneration.

Figure 8.

Decreased osteoclast activity and osteogenic differentiation in T2DM during bone regeneration. (A–C) Gene expressions of mature osteoclast markers (TRAP, Cathepsin K and MMP-9) inside the bone defect region at different time points (3, 7, 14, 28, 56d) during bone regeneration. (D–E) Gene expressions of osteoclast differentiation regulators (RANKL and OPG). (F) RANKL/OPG ratio. (G–H) Gene expressions of osteogenic transcription factors (Runx2 and Sp7), * $p < 0.05$, ** $p < 0.01$.

Table 1.

RT-PCR Primer Sequence

Genes	NCBI reference		Primer Sequence (5'–3')
TRAP	NM_019144.2	Forward	ATGACGCCAATGACAAGAGGTT
		Reverse	GCGCTTGGAGATCTTGGAGTAG
Cathepsin K	NM_031560.2	Forward	GCAGCTAAGTGCAGAGGGTACAG
		Reverse	GGTTCACATTATCACGGTCGC
MMP-9	NM_031055.1	Forward	TCTTCGACTCCAGTAGACAATCCTT
		Reverse	ACTTCCAATACCGACCGTCCT
RANKL	NM_057149.1	Forward	TCGGGTTCCTATAAAGTCAGTC
		Reverse	CAAATGTTGGCGTACAGGTAATAGA
OPG	NM_012870.2	Forward	TTGGCTGAGTGTCTGGTGGA
		Reverse	TCACAGAGGTCAATGTCTTGGATG
Runx2	NM_001278483.1	Forward	GAGAACTACTCTGCCGAGCTACGA
		Reverse	GTGAACTCTTGCCTCGTCCG
SP7	NM_001037632.1	Forward	AGCTGCCTACTTACCCGTCTGA
		Reverse	TGCCCACTATTGCCAACTGC
GAPDH	NM_017008.4	Forward	GCAAGTTCAACGGCACAGTCA
		Reverse	ACGCCAGTAGACTCCACGACA