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Inhibitory Effects of High Glucose/Insulin Environment on Osteoclast Formation and Resorption *in vitro*[*]

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Summary: Patients with type 2 diabetes mellitus (T2DM) exhibit hyperglycemia and hyperinsulinemia and increased risk of fracture at early stage, but they were found to have normal or even enhanced bone mineral density (BMD). This study was aimed to examine the molecular mechanisms governing changes in bone structure and integrity under both hyperglycemic and hyperinsulinemic conditions. Monocytes were isolated from the bone marrow of the C57BL/6 mice, induced to differentiate into osteoclasts by receptor activator of nuclear factor kappa-B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) and exposed to high glucose (33.6 mmol/L), high insulin (1 μmol/L), or a combination of high glucose/high insulin (33.6 mmol/L glucose and 1 μmol/L insulin). Cells cultured in α-MEM alone served as control. After four days of incubation, the cells were harvested and stained for tartrate resistant acid phosphatase (TRAP). Osteoclast-related genes including RANK, cathepsin K and TRAP were determined by using real-time PCR. The resorptive activity of osteoclasts was measured by using a pit formation assay. Osteoclasts that were derived from monocytes were of multinucleated nature and positive for TRAP, a characteristic marker of osteoclasts. Cell counting showed that the number of osteoclasts was much less in high glucose and high glucose/high insulin groups than in normal glucose and high insulin groups. The expression levels of RANK and cathepsin K were significantly decreased in high glucose, high insulin and high glucose/high insulin groups as compared with normal glucose group, and the TRAP activity was substantially inhibited in high glucose environment. The pit formation assay revealed that the resorptive activity of osteoclasts was obviously decreased in high glucose group and high glucose/high insulin group as compared with normal group. It was concluded that osteoclastogenesis is suppressed under hyperglycemic and hyperinsulinemic conditions, suggesting a disruption of the bone metabolism in diabetic patients.
Key words: osteoclastogenesis; glucose; insulin; type 2 diabetes mellitus

Type 2 diabetes mellitus (T2DM) is one of the most prevalent diseases in the world. It is estimated that more than 300 million people were affected by T2DM by the year 2010[1]. In the early stage of this disease, T2DM patients exhibit hyperinsulinemia due to impaired cellular sensitivity to insulin and hyperglycemia, and concomitantly, several complications including osteoporosis and increased fracture occur due to the altered bone metabolism[2]. It is well established that the bone mineral density (BMD) is reduced in type 1 diabetes mellitus (T1DM) patients compared with their healthy counterparts. However, there is no consensus on the changes of the bone metabolism in T2DM patients. A meta-analysis performed by Vestargaard *et al* concluded that the hip fracture risk is increased in both T1DM and T2DM patients, whereas BMD is increased in T2DM and decreased in T1DM[3]. However, other investigators demonstrated that the BMD of T2DM rats is decreased when compared with that of non-diabetic rats[4].

Current studies on alterations in the BMD in T2DM predominantly focus on osteoblastogenesis[5-7]. In this study, we investigated the effect of T2DM on bone metabolism from a different perspective, namely osteoclastogenesis. In brief, monocytes were isolated from C57BL/6 mice and cultured *in vitro*. They were induced to differentiate into osteoclasts by using macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL). Meanwhile, they were cultured in high glucose/high insulin conditions to simulate *in vivo* bone marrow microenvironment of T2DM patients. TRAP staining, osteoclast cell counting, real-time PCR analysis and bone resorption assay were used to evaluate the formation and resorptive activity of osteoclasts.

1 MATERIALS AND METHODS

The animal experiment was approved by the Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology, China.

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1.1 Harvest and Culture of Osteoclasts

Bone marrow cells were isolated from the whole bone marrow immediately after euthanasia of C57BL/6 mice. In brief, cells were flushed out of the bone marrow cavity by using a syringe with a 26-gauge needle and collected in the primary culture medium. The cell suspension was drawn through an 18-gauge needle to achieve single cell suspension. Cells were then washed with the primary culture medium and cultured in α-MEM (Hyclone Co., USA) supplemented with 15% heat-inactivated fetal bovine serum (Gibico Co., USA) and 1% penicillin and streptomycin. After 24 h, non-adherent floating cells (i.e. monocytes) were collected and then plated at a density of 5×10^5 cells/well in the 24-well plates. M-CSF (50 ng/mL) and RANKL (50 ng/mL, R&D system, USA) was supplemented to the medium immediately after cell inoculation. Thereafter, cells were exposed to conditions of high glucose (33.6 mmol/L) (Sigma Co., USA), high insulin (1 μmol/L) (Sigma Co., USA) or high glucose and insulin combined (33.6 mmol/L glucose, 1 μmol/L insulin). Those cultured in α-MEM alone served as control. Four days later, cells in different groups were harvested for the following experiments.

1.2 TRAP Staining

Cells in each group were stained for TRAP. Briefly, the culture medium was removed and cells washed with 100 μL PBS. Fixative reagents (50 μL) were then added at room temperature and co-cultured with cells for 5 min. Afterwards, cells was washed 3 times with double distilled water, which was followed by addition of 50 μL chromogenic substrate. Then, cells were incubated at 37°C for a total of 45 min and washed with double distilled water to obtain the images with best quality.

1.3 $TRAP^+$ and Multinucleated Cell Counting

Cell counting was performed on each well of 96-well plates after TRAP staining. Cells which were multinucleated (>3 nuclei per cell) and positive for TRAP were regarded as target cells and then counted.

1.4 Real-time Quantitative PCR

Total RNA was isolated with Trizol (Invitrogen, USA) and used to synthesize cDNA by using the superscript Ⅱ cDNA synthesis kit (Invitrogen, USA). The osteoclast-related genes were respectively assessed by quantitative real-time PCR using SYBR green mastermix (ABI, USA). For real-time PCR reactions, the following primer pairs were used: 18S (5'-TTCGAACGTCTGCCCTATCAA-3', 5'-ATGGTAGGCACGGGGACTA-3'), RANK (5'-CCAGGAGAGGCATTATGAGCA-3', 5'-ACTGTCGGAGGTAGGAGTGC-3'), cathepsin K (5'-GAAGAAGACTCACCAGAAGCAG-3', 5'-CTGTATTCCCCGTTGTGTAGC-3'), TRAP (5'-CACTCCCACCCTGAGATTTGT-3', 5'-CATCGTCTGCACGGTTCTG-3'). The 18S rRNA served as an internal control. The cycling protocols were as follows: 95°C for 3 min, 94°C for 10 s, 60°C for 30 s, for 40 cycles.

1.5 Quantitative Measurement of TRAP Activity

TRAP activity was measured in each group four days after cells were cultured with different concentrations of glucose, insulin or glucose and insulin combined (11.1 mmol/L glucose, 5 μmol/L insulin, 11.1 mmol/L glucose+5 μmol/L insulin; 22.2 mmol/L glucose, 10 μmol/L insulin, 2.2 mmol/L glucose+10 μmol/L insulin; 33.3 mmol/L glucose, 15 μmol/L insulin, 33.3 mmol/L glucose+15 μmol/L insulin). The procedures were performed according to the method as described by Nakasato and coworkers[8]. In brief, P-nitrophenyl phosphate (6.7 mmol/L) was used as the substrate. Cell lysates were mixed with buffer solution (100 mmol/L sodium acetate and 50 mmol/L sodium tartrate) and shaken gently for 10 min. TRAP substrate was added at room temperature and co-cultured with cells for 30 min, and the reaction was stopped by addition of 0.05 mol/L NaOH. The absorbance which represents the TRAP activity was obtained at a wavelength of 405 nm.

1.6 Preparation of Mineral Films

The following procedures were performed as described by Yang and coworkers[9]. Mineral films would be plated in the wells of 48-well plates and the 2-step deposition process was employed. The first step was pre-calcification. The 2.5 times concentrated stimulated body fluid (SBF×2.5) (500 mL) was pipetted into each well to pre-calcify the wells. The SBF×2.5 solution was prepared by mixing buffer, calcium, phosphate solutions at a ratio of 2:1:1. The buffer solution was obtained by dissolving 24.2 g Tris base and 164 mL 1 mol/L HCl in pure MilliQ water to give a total volume of 4 L (final pH=7.4), the calcium solution by dissolving 1.37 mol/L NaCl, 15 mmol/L $MgCl_2{\cdot}6H_2O$ and 25 mmol/L $CaCl_2{\cdot}2H_2O$ in the buffer solution, and the phosphate solution by dissolving 42 mmol/L $NaHCO_3$ and 11.1 mmol/L $Na_2HPO_4.H_2O$ in the buffer solution. SBF×2.5 was refreshed everyday for 3 consecutive days. By day 3, the wells were thoroughly washed with MilliQ water. The second step was to initiate crystal growth, which involved deposition of a crystalline calcium phosphate layer on the pre-calcified plates by pipetting 500 mL of supersaturated calcium phosphate solution (CPS) in each well. The CPS was prepared by dissolving 0.14 mol/L NaCl, 4 mmol/L $CaCl_2{\cdot}2H_2O$, 2.25 mmol/L $Na_2HPO_4{\cdot}H_2O$ and 50 mmol/L Tris in MilliQ water (pH value adjusted to 7.4 by adding HCl) and was refreshed every 3 consecutive days. In the end, the wells were washed with MilliQ water and dried.

1.7 Measurement of Resorptive Activity of Osteoclasts

The osteoclast suspension (500 μL) was seeded into each well of mineral film-coated plates to investigate the resorptive activity of osteoclasts. After cell incubation for 3 h, non-adherent cells were discarded and the adherent cells were subsequently cultured for another 40 h. Then, they were washed with PBS, fixed for 10 min with 4% formaldehyde in PBS and stained for TRAP activity. With each group, four images were obtained randomly under the microscope. The percentage of resorption pit area to the total area was subsequently obtained.

1.8 Statistical Analysis

All the data were expressed as $\bar{x}\pm s$. The statistical analysis was performed using one-way ANOVA with SPSS 13.0. All the experiments were repeated at least three times independently. A P value less than 0.05 was considered to be significantly different.

2 RESULTS

2.1 Osteoclast Cell Counting

TRAP-positive cells were easily seen in all the four

groups (fig. 1A and B). Cells that were of multinucleated nature and positive for TRAP were mature osteoclasts and were counted. It was found that the number of mature osteoclasts was much less in high glucose group than in control group and high insulin group (P<0.05). There was no significant difference in the number of osteoclasts between high glucose group and high glucose/high insulin group (fig. 1C).

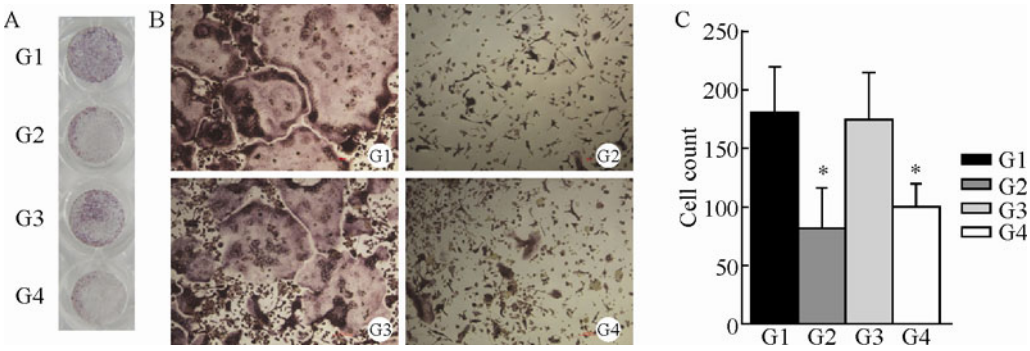

Fig. 1 Cell counting of osteoclasts in different groups

A and B: gross appearance (A) and microscopic view (B) of TRAP-positive cells (×10); C: Counting of osteoclasts. G1: Control group; G2: high glucose group (33.6 mmol/L glucose); G3: high insulin group (1 μmol/L insulin); G4: high glucose/insulin group (33.6 mmol/L glucose and 1 μmol/L insulin). *P<0.05 *vs*. G1

2.3 Osteoclast-related Gene Expression

To further address the phenomenon shown above, the transcriptional changes of osteoclast-related genes (including RANK, cathepsin K and TRAP) were examined. Real-time PCR revealed that the expression levels of the three osteoclast-related genes were significantly decreased in high glucose, high insulin and high glucose/high insulin groups when compared with the control group (fig. 2). The additive inhibitory effect on expression of the three osteoclast-related genes was shown in the high glucose/high insulin group.

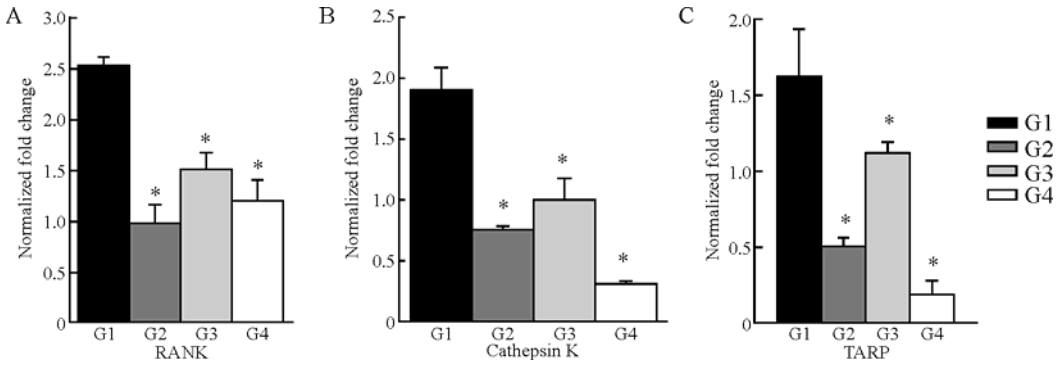

Fig. 2 Real-time PCR analysis of the expression of osteoclast-related genes including RANK (A), cathepsin K (B) and TRAP (C) in different groups

G1: control group; G2: high glucose group (33.6 mmol/L glucose); G3: high insulin group (1 μmol/L insulin); G4: high glucose/insulin group (33.6 mmol/L glucose and 1 μmol/L insulin). *P<0.05 *vs*. G1

2.4 TRAP Activity under Varying Glucose and Insulin Conditions

TRAP activity was measured in cells treated with glucose at different concentrations (11.1, 22.2, and 33.3 mmol/L) and insulin at a concentration of 5, 10 or 15 μmol/L and the combinations of glucose and insulin. It was shown that the TRAP activity was inhibited by glucose in a dose-dependent manner (fig. 3). Insulin at different concentrations (5, 10 or 15 μmol/L) had no significant effects on TRAP activities. The TRAP activity was conspicuously inhibited when cells were treated by glucose and insulin combined. Under glucose/insulin (33.3 mmol/L, 15 μmol/L) condition, the strongest inhibitory effect on TRAP was induced (fig. 3C).

2.5 Mineral Film and Osteoclast Resorptive Activity

The mineral deposition of SBF×2.5 and CPS in 24-well plates resulted in a uniform crystalline white calcium phosphate film (fig. 4A). The area where mineral deposition was absent represented resorption pit as indicated by red arrows (fig. 4C). It was found that the percentage of the resorption pit area was significantly decreased in high glucose and high glucose/high insulin groups when compared with control group (P<0.05, fig. 4B and D). Although, the resorption area tended to decrease in high insulin group, there was no significant difference in the percentage of resoprtion pit area between the high insulin group and control group (fig. 4B and D).

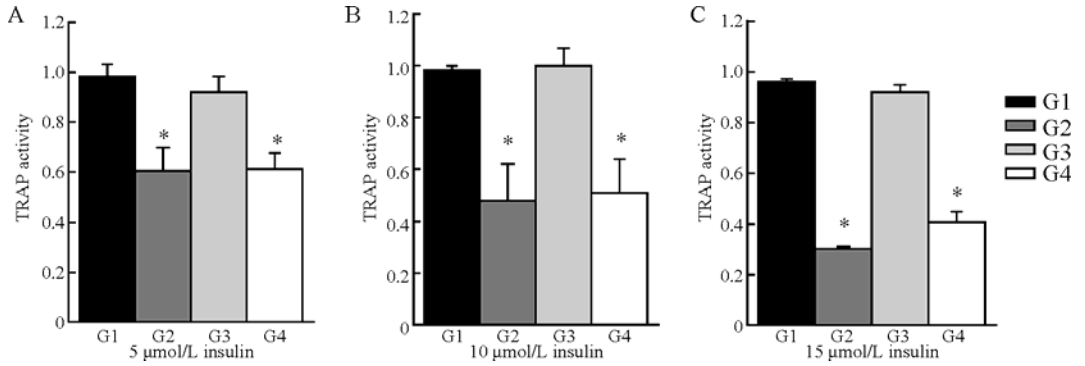

Fig. 3 Measurement of TRAP activity in cells exposed to varying glucose and insulin concentrations

A: G2 (high glucose group, 11.1 mmol/L glucose), G3 (high insulin group, 5 μmol/L insulin), G4 (high glucose/insulin group, 11 mmol/L glucose+5 μmol/L insulin; B: G2 (high glucose group, 22.2 mmol/L glucose), G3 (high insulin group, 10 μmol/L insulin), G4 (high glucose/insulin group, 22.2 mmol/L glucose+10 μmol/L insulin); C: G2 (high glucose group, 33.3 mmol/L glucose), G3 (high insulin group, 15 μmol/L insulin), G4 (high glucose/insulin group, 33.3 mmol/L glucose+15 μmol/L insulin); G1: control group. $^{*}P<0.05$ *vs*. control group

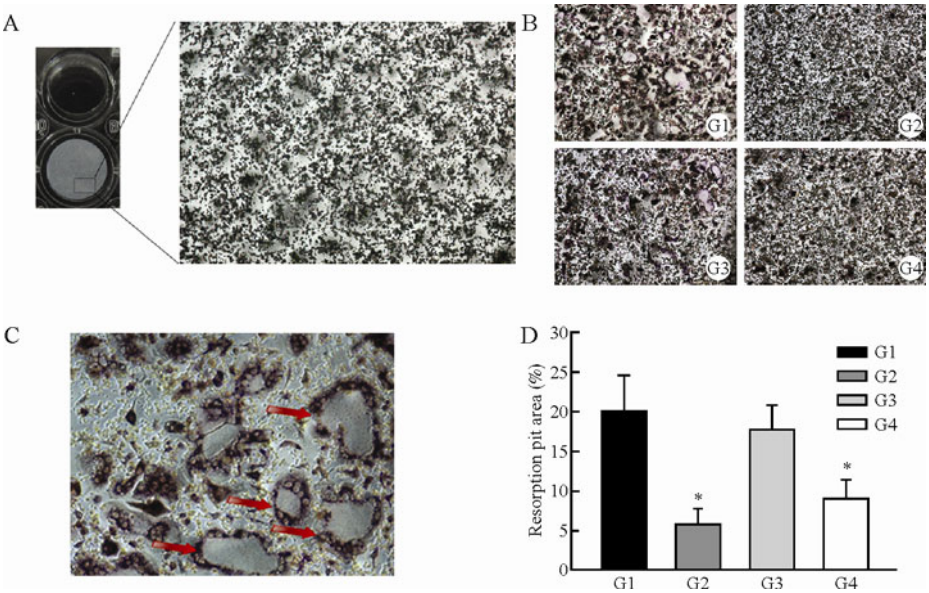

Fig. 4 The pit formation assay for the resorption activity of osteoclasts in different groups

A: Gross appearance and microscopic view of mineral film (×10); B: The microscopic images of osteoclast resorption (×10); C: Resorption pit indicated by red arrows (×40); D: Comparison of the percentage of resorption pit area among different groups; G1: Control group; G2: high glucose group (33.6 mmol/L glucose); G3: high insulin group (1 μmol/L insulin); G4: high glucose/insulin group (33.6 mmol/L glucose and 1 μmol/L insulin). $^{*}P<0.05$ *vs*. G1

3 DISCUSSION

Patients with T2DM have normal-to-higher BMD than non-diabetics. However, they are at a 2-fold high risk for fracture despite higher BMD[10]. This paradox between normal or higher BMD and increased fracture risk in T2DM has prompted many researchers to investigate the association between diabetes-related factors and bone metabolism.

In the early stage of T2DM, patients exhibit hyperinsulinemia due to impaired cellular sensitivity to insulin and hyperglycemia due to insulin resistance. The high glucose/high insulin environment plays an important role in bone metabolism in T2DM patients. In this study, we focused on the effects of high glucose/high insulin on osteoclastogenesis to better understand the bone metabolism in T2DM. Monocytes were induced by RANKL and M-CSF to differentiate into osteoclasts which are multinucleated and positive for TRAP. Our results showed that the number of osteoclasts was significantly decreased, the expression levels of osteoclast-related genes were conspicuously reduced, the TRAP activity was substantially inhibited and the osteoclast resorption activity was suppressed under hyperglycemic and hyperinsulinemic conditions which are similar to the diabetic internal conditions.

The skeleton houses hematopoietic cells and acts as a reservoir for calcium and phosphate[11]. In the adult skeleton, the bone resorption carried out by osteoclasts and bone formation by osteoblasts are in balance so that the structure and integrity of the bone remain homeo-

statically controlled. Maintenance of BMD requires the balance of function between osteoblasts and osteoclasts[12]. Studies have revealed that the bone is constantly remodeled throughout life and the process of bone remodeling is regulated by both local and systemic factors[13-15].

The effects of high glucose/high insulin environment on osteoblasts have been extensively studied. Gopalakrishnan and his colleague reported high concentrations (16.5 and 49.5 mmol/L) of glucose significantly inhibited the proliferation of bone mesenchymal stem cells in a dose-dependent manner, as reflected by cellular thymidine incorporation[16]. Zhen *et al* revealed that differentiation of primary rat calvarial osteoblasts was decreased by exposure to high concentrations of glucose (between 22–44 mmol/L) and high glucose environment may affect the osteoblastic function via Runx2, IGF-1, IGF-1R genes[17]. Moreover, it was found that the proliferation of primary human osteoblasts was increased upon stimulation with glucose and insulin, likely due to an increase energy balance[18]. The effect of insulin on osteoblasts is through a tyrosine kinase, named insulin receptor (IR), which is abundant in osteoblasts[19]. However, little is known regarding the effects of high glucose/high insulin environment on osteoclast differentiation and function, and the link between osteoblast-osteoclast interaction and bone turnover in T2DM is not carefully examined.

Osteoclasts are the principal bone resorbing cells of the body and derived from a hematopoietic precursor of the monocytic lineage common to macrophages and myeloid dendritic cells (DCs). They are characterized by high expression of TRAP and multiple nuclei in morphology. RANK, a type of TNFR, is a central activator of NF-κB. RANK expression provides a necessary and specific signal for the differentiation of myeloid-derived osteoclasts. In the present study, we found that the number of osteoclasts was significantly reduced in high glucose and high glucose/high insulin groups, as evidenced by a profound decrease in the number of TRAP-positive multinucleated cells; the mRNA expression levels of RANK and TRAP and the TRAP activity were substantially decreased in high glucose and high insulin/high glucose groups. These results suggested the suppression of RANKL- and M-CSF-induced osteoclast formation under hyperglycemic and hyperinsulinemic conditions, which is consistent with the findings reported by Wittrant *et al* who found that high D(+) glucose inhibits RANKL-induced TRACP activity and osteoclast differentiation[20]. But it was noted that we failed to observe the inhibitory effect of high insulin on the number of osteoclasts and TRAP activity, and the synergistic inhibitory effects of high glucose and high insulin on osteoclast formation. Huang *et al* found that L-SACC1 mice, a murine model of impaired insulin clearance in liver causing hyperinsulinemia and insulin resistance without fasting hyperglycemia, exhibited reduced number of osteoclasts and concluded that high levels of insulin affect recruitment and differentiation of osteoclasts by impairing the RANKL signaling pathway[21]. The discrepancy caused by our study might lie in that we employed an *in vitro* system and treated monocytes with insulin at a different concentration. It is necessary to use an *in vivo* system to further determine the effect of high insulin on osteoclast formation in the future.

Cathepsin K is a cysteine protease expressed predominantly in osteoclasts. Bone resorption depends upon the synthesis of cathepsin K. In this study, the osteoclast function was examined by detecting the expression of cathepsin K and the resorptive activity of osteoclasts. The results showed that the expression of cathepsin K and the percentage of resorption pit area were significantly decreased in high glucose and high glucose/high insulin groups, which suggested that the function of osteoclasts is impaired under hyperglycemic and hyperinsulinemic conditions.

In conclusion, our study demonstrated osteoclastogenesis is significantly inhibited in *in vitro* high glucose/high insulin environment. The changes in the formation and resorption activity of osteoclasts under hyperglycemic and hyperinsulinemic condition may lead to the change in bone mass seen in patients with T2DM. However, further studies on both osteoclastogenesis and osteoblastogenesis are needed to provide more insights into the changes in BMD in T2DM patients.

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