Effects of metformin on the prevention of bisphosphonate-related osteonecrosis of the jaw-like lesions in rats

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Abstract

Purpose: In this study, we aimed to investigate the effect of glucose metabolism on bone healing after tooth extraction in an osteoporosis rat model administered zoledronic acid (ZA) and dexamethasone (DX).

Methods: In total, 24 male Wistar rats (4 weeks old) were randomly assigned to four groups: Control (subcutaneous physiological saline), ZD (subcutaneous ZA and DX twice a week), Ins+ZD (subcutaneous insulin followed by ZD treatment), and Met+ZD (oral metformin followed by ZD treatment). Blood was collected every two weeks. Two weeks after treatment initiation, the first molar tooth on the right maxilla was extracted from all rats. Four weeks later, the rats were sacrificed, and bone healing was assessed. Maxillae samples were fixed and scanned using micro-computed tomography for quantifying areas of bone defects. Hematoxylin-eosin and tartrate-resistant acid phosphatase (TRAP) staining were performed to evaluate bone apoptosis and osteoclast number.

Results: In all experimental groups, body weight was statistically lower than that in the Control group, with no changes observed in uncarboxylated osteocalcin concentrations. The radiological analysis revealed that insulin or metformin administration improved healing in the tooth extraction socket (p < 0.01). Histological examination revealed that the osteonecrosis area was reduced in the Ins+ZD and Met+ZD groups (p < 0.01). TRAP staining presented increased osteoclast numbers in the ZD group when compared with that observed in the Control.

Conclusions: Tooth extraction with long-term ZA and DX administration inhibited bone remodeling and induced bisphosphonate-related osteonecrosis of the jaw-like lesions. Metformin exerted protective effects against osteonecrosis of the jaw.

Keywords: Dental implant, Bone healing, MRONJ, Glucose metabolism

1. Introduction

Bone fracture is a serious health problem, with a high risk related to various diseases, such as osteoporosis and diabetes [1]. Along with a growing incidence of osteoporosis in the elderly, the number of patients with osteoporosis is annually increasing. Hence, countermeasures for treating bone fractures are crucial in medical treatment, remaining a socially relevant issue. Antiresorptive medications, including denosumab or bisphosphonates (BP), are used as first-line selective drugs to reduce skeletal-related events, as well as to lower the risk of breast cancer metastasis to bones [2,3]. Conversely, it has been reported that osteonecrosis of the jaw occurs after tooth extraction in patients with a history of osteoporosis and cancer treatments [4].

Recently, this occurrence has been termed as medication-related osteonecrosis of the jaw (MRONJ), as it is also known to develop in patients receiving other antiresorptive medications [5]. Even without tooth extraction, insertion of dental implants during or after treatment with BP accelerates MRONJ progression [6,7].

Currently, preventive measures available against MRONJ include improving oral hygiene, discontinuing the administration of antiresorptive medications, and avoiding dentoalveolar surgical procedures. Moreover, following dentoalveolar surgery in osteoporosis patients undergoing BP treatment, a decrease in MRONJ occurrence has not observed, even in the washout group [8].

Although the onset mechanism of MRONJ remains elusive, the following have been speculated as possible contributing factors: osteoclast suppression, increase in oral bacterial infection, inhibition of angiogenesis, reduced blood flow, decrease in immunity, and inhibition of epithelial cell migration [9]. Collectively, it remains a problem that the administration of antiresorptive medications poses a risk in dental treatment.

Recently, the close relationship between bone and glucose metabolism has been reported. Previous studies have revealed that patients receiving BP demonstrate significantly higher fasting blood glucose levels [10]. Reportedly, patients with MRONJ develop diabetes...
mellitus [11]. In vitro, the bone matrix protein, osteocalcin, increases insulin secretion from pancreatic beta cells to regulate glucose metabolism [12]. In contrast, the activation of the insulin receptor in osteoblasts accelerates bone remodeling [13,14]. According to current guidelines, metformin, a biguanide, remains the first-choice oral agent for the treatment of diabetes [15]. Furthermore, metformin may stimulate osteoblast differentiation through the transactivation of genes via AMP-activated protein kinase (AMPK) regulation. Reportedly, AMPK significantly increases the expression of important osteogenic genes such as osteocalcin and alkaline phosphatase [16].

Notably, there exists a high possibility that glucose and bone metabolism are positively correlated with each other [17]. This relationship can present an effective strategy to allow dental treatment, with surgical invasion, in patients presenting abnormalities in bone metabolism. Therefore, in this study, we aimed to investigate the effect of antidiabetic drugs on bone metabolism using a rat model of osteoporosis.

2. Materials and methods

2.1. Animals

Twenty-four male Wistar rats (4 weeks of age) were provided water and a standard diet. The body weight was measured once a week until the rats were 10 weeks old. Next, the animals were divided into four groups: Control [subcutaneous injection of physiological saline], ZD [subcutaneous injection of zoledronic acid (ZA) 0.1 mg/kg and dexamethasone (DX) 1 mg/kg twice a week], Ins+ZD [subcutaneous injection of insulin (4 to 6 IU) followed by ZD treatment], and Met+ZD (oral metformin 250 mg/kg followed by ZD treatment), with six animals (n = 6) in each group. The experimental protocol was approved by the committee for the use and management of the Animal Committee of Kyushu Dental University (No. 18-005).

2.2. Specimen preparation

Triple mixed anesthetics were prepared using medetomidine (0.4 mg/kg), midazolam (4.0 mg/kg), and butorphanol (5.0 mg/kg). Under general anesthesia following intraperitoneal injection, tooth extraction was performed at 6 weeks of age. The first molar on the right maxilla from each rat was extracted using a forceps and curette. Extraction was performed at 6 weeks of age. The first molar on the right maxilla from each rat was extracted using a forceps and curette. After experiment initiation, blood (5 μL) was collected from the external tarsal vein of rats at two-week intervals. Blood samples were centrifuged (1200 × g for 20 min) to separate the serum, and serum was measured over time until the end of the experiment. The body weight and serum GluOC levels were measured. (Fig. 1).

2.3. Enzyme-linked immunosorbert assay (ELISA) assay of uncarboxylated osteocalcin (GluOC) protein levels.

After experiment initiation, blood (5 μL) was collected from the external tarsal vein of rats at two-week intervals. Blood samples were centrifuged (1200 × g for 20 min) to separate the serum, and serum samples were stored at -28°C. Venous blood was collected and ELISA was performed to detect serum GluOC (rat Glu-OC kit; Takara Bio, Kusatsu, Japan).

2.4. Micro-computed tomography (μCT)

The collected maxillae were subjected to μCT using Cosmo Scan FX (Rigaku, Tokyo, Japan). Maxilla specimens were scanned in sections of 5 μm thickness and the generated images were analyzed using an image analysis software (DentsplySirona, Tokyo, Japan), with the healing status of the tooth extraction socket observed buccolingually and mesiodistally. The cross-sectional buccolingual area was measured at 5 points in intervals of 0.5 mm, including the 1.0 mm distal part from the upper second molar neck. The cross-sectional mesiodistal area was measured at 5 points in intervals of 0.2 mm, including the cross-section connecting the deepest part of the extraction socket of two roots of the palate. On buccolingual observation, the area of bone defect below the line connecting the buccal alveolar bone apex and the lingual alveolar bone apex, in the tomographic image of the tooth extraction socket, was measured. On mesiodistal observation, the area of the bone defect under the line connecting the apex of the mesial bone and apex of the distal bone of the tooth extraction socket was measured. (Fig. 2).

2.5. Histopathology analysis

The collected maxillae were fixed using a 10% formalin solution. After fixation, the demineralized maxillae were embedded in paraffin. Thereafter, the samples were sliced into 5 μm thickness in the sagittal direction of the tooth extraction socket. Hematoxylin and eosin (H&E) staining and tartrate-resistant acid phosphatase (TRAP) staining were performed using standard staining protocols for maxilla samples. The range of osteonecrotic area, the number of empty lacunae, osteocyte density, and polymorphonuclear cells were evaluated using H&E staining, and the number of osteoclasts presenting TRAP-positive multinucleated cells in the tooth extraction socket was measured. In accordance with a previous report, the osteonecrotic area was defined as empty lacunae of > 500 mm² [18]. The measurements were performed using a VHX-5000 (KEYENCE, Tokyo, Japan).

2.6. Statistical analysis

Data were analyzed using analysis of variance (ANOVA) and the Tukey-Kramer test. The results are expressed as mean ± standard error. For all analyzed data, the significance level was determined as a p-value of < 0.05.

3. Results

3.1. Body weight and serum GluOC

For each group, the body weight and serum GluOC levels were measured over time until the end of the experiment. The body weight was significantly lower in the ZD, Ins+ZD, and Met+ZD groups than in the Control group (p < 0.05; Fig. 3A). Furthermore, body weights decreased following ZA and DX administration and failed to improve following insulin or metformin treatment. A chronological change was detected in the GluOC level, a marker for bone metabolism. No difference was observed in the serum GluOC levels between all groups (Fig. 3B).
Fig. 2. (A) The tooth extraction socket was observed at five areas in each direction. (B) Results of buccolingual observation. (C) Results of mesiodistal observation.

Fig. 3. (A) Changes in body weight between the Control, ZD, Ins+ZD, and Met+ZD groups during a 42-day period. Body weight was measured once per week. ** p < 0.01. (B) Serum levels of GluOC in the Control, ZD, Ins+ZD, and Met+ZD groups, as measured by ELISA.

3.2. Assessment of mucosal and socket healing

Following macroscopic examination, the findings demonstrated that the oral mucosa was healed in the Control, Ins+ZD, and Met+ZD groups 4 weeks after tooth extraction. The oral mucosa was not completely healed, with exposed bone observed in the tooth extraction socket of 4 rats in the ZD group (Fig. 4A). The healing state of the tooth extraction socket was evaluated using μCT in buccolingual and mesiodistal directions (Fig. 4B, C). Compared with the Control group, the tooth extraction socket in the ZD group failed to heal; in contrast, compared with the ZD group, improved healing was observed in the Ins+ZD and Met+ZD groups (p < 0.05; Fig. 4D, E). No difference was observed in the bone healing state between the Ins+ZD and Met+ZD groups. 

3.3. Histologic analysis

The osteonecrotic area was examined around the tooth extraction socket. In the ZD group, the osteonecrotic area was largely wider than that observed in the Control group; however, osteonecrotic areas in the Ins+ZD and Met+ZD groups decreased to the same width as that in the Control group (p < 0.05; Fig. 5A, B). Compared with all groups, the ZD group demonstrated higher necrotic bone fractions and more empty lacunae (p < 0.01; Fig. 5C). Conversely, the osteocyte density was lower in the ZD group than in the Control and Met+ZD groups (p < 0.01; Fig. 5D). In the ZD group, the number of polymorphonuclear cells was higher than that in the Control group (p < 0.05; Fig. 5E). Additionally, TRAP staining was performed to evaluate the number of osteoclasts. In the ZD group, the number of TRAP-positive cells was notably higher than that observed in the Control group (p < 0.05; Fig. 5C, D). No significant differences were observed in the number of TRAP-positive cells between the ZD, Ins+ZD, and Met+ZD groups.

4. Discussion

Although antiresorptive medications such as BP are effective in the treatment of patients with osteoporosis and cancer and are commonly prescribed, several studies have reported that the administration of antiresorptive drugs increases the risk of developing MRONJ [19,20]. Furthermore, numerous studies have attempted to elucidate the underlying mechanism of MRONJ, but its pathophysiology remains poorly understood [9].

Recent studies have revealed a close relationship between bone and glucose metabolism [21-23]. The bone matrix protein, osteocalcin, is known to enhance the expression of glucagon-like peptide 1 (GLP-1), a peptide hormone secreted from the small intestine mucosal epithelium, that reportedly improves arteriosclerosis and cardiac function [24,25]. Thus, the existence of systemic organ networks recently has become more evident.

In this study, body weight loss was observed in three groups receiving ZD. Long-term DX administration inhibits food intake in rats fed a less-palatable diet, which can reduce body weight [26]. Furthermore, the severity of stress directly induces body weight loss [27]. Here, loss of body weight in the ZD group, as well as in the Ins+ZD and Met+ZD groups, was attributed to the effects of daily administered medication and tooth extraction. Our result showed that no significant difference existed between the four groups in serum GluOC concentrations, a bone metabolism marker. Previously, a study has presented differences in blood concentrations of other bone metabolism markers including GluOC; however, other studies have demonstrated results similar to our findings [28,29]. Several factors, including individual variation, diurnal variation, dietary variation, and urinary marker creatinine correction, reportedly affect bone metabolism markers in the blood and urine. Furthermore, it is challenging to use...
In the present study, delayed healing of the tooth extraction sockets was quantified. * p < 0.05. (F) Representative images of TRAP-stained tooth extraction sockets (arrow: TRAP-positive cells). Scale bar = 100 μm (TRAP staining; original magnification, 300×). (G) TRAP-positive cells in tooth extraction sockets were quantified. * p < 0.05.

only GluOC as a reliable marker to determine the time point of surgical intervention after BP withdrawal. However, no difference was observed between the control and MRONJ groups, even when markers P1NP, TRACP-5b, and C-terminal cross-linked telopeptides of type I collagen (CTX) were measured, and further studies combining GluOC with novel markers are required [30,31]. Local bone resorption/formation events could not be presented as these bone metabolism markers reflect whole-body changes. Root fracture was less likely to occur during tooth extraction probably because the jawbone of 6-week-old rats is flexible.

In the present study, delayed healing of the tooth extraction membrane and exposed bone were observed with ZD administration; however, the administration of ZD with insulin or metformin improved mucosal healing. Abnormalities in bone metabolism affect the regulation of global glucose homeostasis [32]. It is postulated that a diabetes-like condition occurs and, as a result, wound healing is delayed. The results of the present study are supported by a previous report that demonstrated improved epithelial healing following the administration of metformin in a diabetic rat model presenting cleft wounds on the epithelium [33]. Moreover, there are various risk factors for the onset of MRONJ. Reportedly, an oral bacterial infection on an open wound induced by tooth extraction is one risk factor [34]. DX, an immunosuppressant, could weaken the host response to bacteria colonizing the bone and increase the probability of bone infection and exposure. A high concentration of DX alone could induce MRONJ. In the present study, DX was administered at a dose of 1.0 mg/kg twice weekly, a concentration that reportedly affects neither bone metabolism nor body weight [35]. Data, such as the number of osteoclasts, osteocyte density, and infiltration of polymorphonuclear cells, are necessary to define BRONJ (bisphosphonate-related osteonecrosis of the jaw). However, we defined the extent of jaw osteonecrosis by using a method based on previous reports [18], followed by analysis. Reportedly, the incidence of BRONJ in the control group was 66%, equivalent to that observed in a previous report [36]. Although it remains unclear whether BRONJ was developed in the experimental model before insulin administration, the rate of bone exposure was 66% in the control group and 0% in the experimental group, suggesting that antidiabetic drugs are an effective treatment for BRONJ.

\[ \text{Fig. 5.} \ (A) \text{Representative images of H&E-stained tooth extraction sockets from each group. The areas surrounded by black line are osteonecrotic areas (arrow e: empty lacunae, arrow p: polymorphonuclear cells). Scale bar = 100 μm (H&E staining; original magnification, 200×). (B) Osteonecrosis in maxilla specimens was quantified. ** p < 0.01. (C) The number of empty lacunae was quantified. * p < 0.05. (D) Osteocyte density was quantified. * p < 0.05, ** p < 0.01. (E) The number of polymorphonuclear cells was quantified. * p < 0.05. (F) Representative images of TRAP-stained tooth extraction sockets (arrow: TRAP-positive cells). Scale bar = 100 μm (TRAP staining; original magnification, 300×). (G) TRAP-positive cells in tooth extraction sockets were quantified. * p < 0.05.} \]
risk of MRONJ increases by 16.4-fold following tooth extraction in patients undergoing BP treatment when compared with that in healthy patients [44]. Furthermore, another study has reported that the risk factor for MRONJ is not the tooth extraction itself, but the failure of the patient's intraoral cleaning conditions that can present a major risk factor [45]. Furthermore, it has been demonstrated that tooth extraction, if the tooth needing extraction is infected (such as in the case of periapical periodontitis), exacerbates the occurrence of MRONJ [46]. In our study, BP-related osteonecrosis of the jaw-like lesions was observed in the ZD group after tooth extraction, but the osteonecrotic area was decreased following the administration of insulin or metformin, affecting bone metabolism. Insulin and its signaling pathway play an essential role in osteoblast differentiation, collagen synthesis, and bone formation. Several preclinical studies have shown that metformin enhances the differentiation of stromal cells into osteoblasts by increasing Runx2 expression, inhibiting differentiation into adipocytes by decreasing peroxisome proliferator-activated receptor gamma (PPARγ) expression [47]. Furthermore, metformin increases the differentiation and mineralization of osteoblasts and directly suppresses osteoclast activity [48,49]. Reportedly, ZA causes MRONJ by affecting macrophages, T cells, and the other cells in the body [50]. Based on its action in these cells, metformin may stimulate osteoblastic differentiation through the transcriptional activation of genes via AMP-activated protein kinase (AMPK) regulation. Hence, activation of bone turnover may be improved. Notably, antidiabetic drugs do not directly inhibit BRONJ induced by ZD, but instead indirectly reduce osteonecrosis. The insulin preparation, used as a positive control in this study, is an effective treatment for diabetic patients, but induces severe hypoglycemic symptoms when administered to non-diabetic patients. Therefore, metformin, which can be used safely without directly promoting insulin secretion and can be administered orally, is considered a beneficial therapeutic agent.

The limitation of this study is that the metformin and insulin monotherapy groups were not included. Although metformin monotherapy reportedly improved bone loss in a rat model of periodontitis [51], further studies including metformin monotherapy and insulin monotherapy are needed to clarify the effects of these drugs on bone metabolism and systemic conditions. Another limitation was the diagnostic methods utilized for MRONJ. The extent of jaw osteonecrosis, determined as previously reported, was employed as a diagnostic criterion. However, histomorphometric analyses of the number of empty lacunae, osteocyte density, and infiltration of polymorphonuclear cells could not be performed. Therefore, in the present study, MRONJ was replaced by BP-related osteonecrosis of the jaw-like lesions.

5. Conclusion

An antidiabetic drug was experimentally administered to rats presenting delayed wound healing in the jawbone caused by BP administration, revealing the following results. Metformin, which can be administered orally, affected bone metabolism and promoted wound healing in rats treated with BP. These results suggest that drug therapy can control the risk for MRONJ onset associated with surgical dental treatments, such as dental implant and tooth extraction.

Conflict of interest statement

The authors declare no conflicts of interest associated with this manuscript.

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