Pyridoxamine improves diabetes-evoked delayed bone repair in mice

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Abstract
Diabetes mellitus is associated with a risk of delayed bone healing and pseudoarthrosis. The production of highly reactive intermediate derivatives of glucose metabolism, including methylglyoxal (MGO), and subsequent acceleration of the glycation reaction under chronic sustained hyperglycemia are implicated in diabetic complications. Pyridoxamine (PM), a natural form of vitamin B6, has been reported to inhibit glycation and proposed to be effective in restraining the complications of diabetes. In the present study, we examined the effectiveness of PM treatment in disturbed bone healing in diabetic mice and MGO-induced osteoblast dysfunction. Multiple injections of low-dose streptozotocin (STZ) (50 mg/kg body weight for 5 days) were injected to induce diabetes in mice. After confirming overt hyperglycemia, a drill-hole injury (5.0 mm) was introduced into the mouse femur and bone defect healing was evaluated with or without PM treatment. Although the diabetic status was not affected by PM treatment, computed tomography scanning images revealed that delayed bone healing was significantly improved in the early phase on days 3 and 10 after injury; the result is also supported by a histological evaluation. In vitro cell-based experiments showed that the osteoblastic differentiation in MC3T3 cells was significantly inhibited by MGO exposure in a dose-dependent manner, but the impairment was recovered by PM treatment. In conclusion, PM, a water-soluble vitamin, could be a useful candidate for recovery against bone injury induced by MGO toxicity in diabetes.

Introduction
Diabetes mellitus is a metabolic disorder, and its prevalence is increasing worldwide. It is a major cause of cardiovascular disease, stroke, chronic kidney disease, neuropathy, blindness, and cancer, which are closely associated with morbidity and mortality in patients with diabetes. Diabetes has also been recognized to affect bone health in terms of bone strength, turnover, mineral density, and structure. Delayed bone healing is recognized as an additional complication of longstanding diabetes, which is associated with a high risk of delayed union, non-union, or pseudoarthrosis [1-3]. Prevention and improvement of the disabling bone complications in diabetes have attracted more attention. Considering the underground mechanisms of hyperglycemia, glycate stress and oxidative stress are known to be associated with the development of diabetic complications including delayed bone healing due to reduced osteoblast differentiation, increased osteoclast activity, and induction of apoptosis in chondrocytes and osteoblasts [4-7]. Glycative stress is an overwhelming and unfavorable glycation state that has been established as a pathogenic factor. Glycation is a non-enzymatic reaction that ultimately results in the formation of advanced glycation end-products (AGEs) in biological macromolecules. The characteristic increase in glycate stress under high glucose conditions in vivo leads to diabetic complications due to accumulation of AGEs from exogenous and endogenous sources and the production of dicarbonyls, which are reactive compounds and major AGE precursors. The importance of glycative stress is proved by the fact that hemoglobin A1c (HbA1c), a compound with an Amadori rearrangement produced during early glycation reaction, is closely correlated with the development of diabetic complications, and is clinically used as a surrogate marker of an average blood glucose level over approximately one month [8-10]. AGEs and dicarbonyls are recognized as pro-inflammatory and pro-oxidant mediators that lead to various biological responses, predominantly by activating the receptor for AGEs.

We have previously reported that glycative stress and accumulation of AGEs elicit delayed bone healing in mice [11]. The most reactive dicarbonyl, methylglyoxal (MGO), inhibits osteoblast differentiation [11]. Thus, the production of MGO should be inhibited to elucidate its beneficial role against bone problems in diabetes. Currently, pyridoxamine (PM) is receiving considerable attention as an AGE inhibitor and an investigational drug for the treatment of diabetic complications. PM is one of the three natural forms of vitamin B6, together with pyridoxal (PL) and pyridoxine (PN), and has been identified as an anti-glycating agent that act via trapping of α-dicarbonyl compounds [12-21]. Previous studies have shown that PM inhibits the formation of AGEs and retards the development of...
diabetic nephropathy and retinopathy in animal models of diabetes [22]. In the present study, we aimed to examine the effectiveness of PM treatment in improving bone repair ability in vivo using hyperglycemic diabetic mice, along with restoration of osteoblast differentiation in vitro.

Materials and methods

Cell culture

Mouse MC3T3 cells (Riken Cell Bank, Tsukuba, Japan) were maintained in α-minimum essential medium (5.5 mmol/L glucose; Wako Pure Chemical Industries, Osaka, Japan), supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in a 5% CO₂ atmosphere at 37 °C. To induce osteoblastic differentiation in MC3T3 cells, 10 mM β-glycerophosphate, 0.1 μM dexamethasone, and 50 μM ascorbic acid were added to the cell culture medium. For the assays, 0.5 – 2.0 mM MGO (Sigma Aldrich) and 0.3 – 3.0 mM pyridoxamine-dihydrochloride-monohydrate (4-aminomethyl-3-hydroxy-2-methyl-5-oxymethylpyridiinhydrochloride, Tokyo chemical industry, Japan) was added to the differentiation medium, and the medium was changed every other day.

Alkaline phosphatase (ALP) activity assay

ALP activity in MC3T3 cells was determined 7 days after the induction of osteoblastic differentiation, using a TRACP & ALP Assay kit (Takara Bio, Inc., Otsu, Japan), according to the manufacturer’s protocol.

Experimental animals and induction of diabetes

Male C57BL/6j mice (weight, approximately 21 g) at 6 weeks of age were purchased from Charles River Japan, Inc. (Yokohama, Japan). They were acclimatized for 1 week prior to the start of the experiment. For diabetes induction, streptozotocin (STZ; 50 mg/kg body weight) was intraperitoneally injected daily for 5 days according to the Animal Models of Diabetic Complications Consortium (AMDCCC) protocols. In contrast, sodium citrate buffer was injected into mice of non-diabetic control group. PM was administered to the animals in drinking water (0.9 mmol/L), which was given to mice in the drinking water. We first evaluated the effects of PM treatment on the bone defect repair ability in vivo using hyperglycemic diabetic mice, along with restoration of osteoblast differentiation in vitro. PM was administered to the animals in drinking water (0.9 mmol/L), which was given to mice in the drinking water. We first evaluated the effects of PM treatment on the bone defect repair ability in vivo using hyperglycemic diabetic mice, along with restoration of osteoblast differentiation in vitro.

Computed tomography (CT) scanning

Whole femur bones were scanned under anesthetized condition using an X-ray CT system (Latheta LCT 200; Hitachi Aloka Medical, Tokyo, Japan) at 0, 3, 7, 10, and 14 days after the drill hole injury. The healing process in the bone defect lesions was evaluated using a suitable analysis software (AzeWin; AZE, Ltd., Tokyo, Japan). Images were processed in a multiplanar reconstruction, according to oblique coronal planes, maintaining the working axes parallel to the center line of the bone defect. CT values at the area of the bone defect were calculated at every phase, as described previously [11].

Histopathological examination

The left femur was extracted and fixed in 10% buffered formaldehyde solution, 7 days after the drill hole injury. After 12 h of fixation, soft tissues were cleaned, and specimens were decalcified in formic acid sodium nitrate solution as described previously. The specimens with a drill hole were sectioned and embedded in paraffin. Mid-sagittal serial sections (5 to 7 μm thick) were prepared and stained with hematoxylin and eosin (H&E). Histological sections were examined using light microscopy at a magnification of 100×.

Statistical analysis

Data are presented as the mean ± standard error of the mean. The differences in the measured variables among the four groups were analyzed using non-repeated one-way analysis of variance (ANOVA). Post hoc multiple comparison using Tukey’s method was performed to assess differences between the groups. Differences with a p value < 0.05 were considered significant. Statistical analysis was performed using the SPSS software (IBM SPSS Statistics Version 22, IBM, Armonk, NY, USA).

Results

Effects of PM treatment on bone defect repair in STZ-induced diabetic mice

To examine the effectiveness of PM treatment on bone defect repair in diabetes, DM and CNT were used with or without PM treatment (1 g/L), which was given to mice in the drinking water. We first evaluated hyperglycemic conditions related to diabetes. As a result, overt diabetic status was observed in STZ-induced DM compared to CNT: body weight (22 ± 1.4 vs. 27 ± 1.7 g), non-fasting blood glucose levels (531 ± 94.8 vs. 132 ± 10.6 mg/dl), and HbA1c (7.9% ± 0.99% vs. 3.7% ± 0.28%) (p < 0.05) at 3 weeks after multiple low-dose STZ injections (50 mg/kg body weight, daily, for 5 days) (Table 1). Polydipsia was evident in DM group, compared to CNT group (9.3 ± 0.86 vs. 3.3 ± 0.22 g/day; DM vs. CNT) (Table 1). PM treatment did not affect the diabetic status of DM group (body weight, 22 ± 1.4 g vs. 22 ± 1.7 g; body glucose, 531 ± 94.8 vs. 539 ± 63.0 mg/dl; HbA1c, 7.9% ± 0.99% vs. 7.7% ± 0.36%; water intake, 9.3 ± 0.86 vs. 6.6 ± 3.82 g/day; DM vs. DM+PM, respectively) (Table 1). In addition, PM did not change any indices of CNT group (Table 1).
Further, we investigated bone defect repair of drill hole defects in the mouse femur, which was evaluated using CT scanning. CT images revealed delayed bone repair in the drill hole lesions in DM group compared to CNT and DM+PM groups (Figure 1A). Quantitative evaluations demonstrated that the CT values of the bone defect portion in DM group were significantly lower than those in CNT group, even at 3 days after the injury (Figure 1B). We observed significantly impaired bone healing in DM group compared to the CNT group, during all observation periods (3, 7, 10, and 14 days) (Figure 1B). However, PM treatment significantly improved bone healing in DM group at 3, 7, and 10 days after the drill hole injury (Figure 1B). Moreover, a negative correlation between individual HbA1c and CT values at 7 days after the injury, was obtained for DM group (Figure 2), indicating that elevated level of glycated HbA1c could be associated with delayed bone healing and low CT values. However, PM treatment (DM+PM) group did not show a negative correlation (Figure 2).

Furthermore, histological findings also showed that the bone hole defect was not repaired in DM group, compared to other groups in the restoration stage at 10 days after the injury (Figure 3). PM-treated (DM+PM) group showed recovered bone hole filled with new bone tissues, as seen in the CNT and CNT+PM groups (Figure 3).

**Effects of PM treatment on MGO-induced deterioration of osteoblastic differentiation of MC3T3 cells in vitro**

*In vitro* cell-based assays demonstrated that exposure to MGO inhibited osteoblastic differentiation in MC3T3 cells, as indicated by ALP activity, in a dose-dependent manner (Figure 4). We performed *in vitro* cell-based assays to evaluate the effectiveness of PM treatment on osteoblastic differentiation and observed significant improvement in MGO-induced deterioration of osteoblastic differentiation in MC3T3 cells (Figure 4).

**Discussion**

The present study is the first to demonstrate the effectiveness of PM treatment in restoration of delayed healing of bone defect in diabetic mice with a femur drill-hole injury (1.0 mm) model (Figures 1 and 3). CT and histological evaluations revealed that the ossification recovery rate of the bone defect lesion in DM+PM group was almost similar to that in CNT group (Figures 1 and 2), although PM treatment was found to have no significant effects on lowering blood glucose or HbA1c levels in STZ-induced diabetic mice (Table 1). Individual data analyses demonstrated that DM mice with higher HbA1c and average blood glucose levels showed lower CT values in the drilled bone defect portion at 7 days after surgery (Figure 2), compared to (DM+PM) group with no negative correlation between HbA1c and CT values. The dosage of PM (1 g/L drinking water; approximately 200 mg/kg/day) used in this study was the same as in our previous report, which proved it to be a safe dose (0.42 μM) that attained serum concentrations of PM within a less toxic range *in vivo*, and its preclinical efficacy has been proven in diabetic kidney disease in KK-Ay/Ta and STZ-induced diabetic rats and mice [17,14]. Notably, PM treatment did not accelerate the ossification recovery in CNT+PM group (Figures 1 and 3), suggesting that PM

![Figure 1. Evaluation of bone defect repair by CT scanning. (A) CT images at 0, 3, 7, 10, and 14 days after bone injury in the left femur. (B) Quantitative evaluation of the CT imaging. A vertical axis indicates CT values of the bone defect lesion. CNT, non-diabetic control groups without pyridoxamine (PM) treatment; CNT+PM, non-diabetic control groups with PM treatment; DM, diabetic groups without PM treatment; DM+PM, diabetic groups with PM treatment; HU, hounsfield unit. Data are presented as the mean ± SEM; n = 10 per group. *, p < 0.05; **, p < 0.01](image-url)
Figure 2. Correlation diagrams between HbA1c and CT values at 3 days after the bone injury. DM, diabetic groups without PM treatment; DM+PM, diabetic groups with PM treatment; n = 10 per group.

Figure 3. Microscopic findings at day 7 after the bone injury. CNT, non-diabetic control groups without PM treatment; CNT+PM, non-diabetic control groups with PM treatment. H&E stain. Magnification, ×10.

Figure 4. In vitro osteoblastic differentiation of MC3T3 cells. (A and B) Alkaline phosphatase (ALP) activity assay using MC3T3 cells at 7 days after the induction of osteoblastic differentiation. Dif, differentiation media; MGO, methylglyoxal; PM, pyridoxamine. Data are presented as the mean ± SEM; n = 6 per group. *p < 0.05.
could affect only diabetes- and hyperglycemia-derived metabolic alterations in the bone repair tissues.

In vitro assay of MC3T3 cells revealed that the addition of MGO into cell culture medium decreased ALP activity, which is the most frequently used marker for osteoblast differentiation and functions (Figure 4). However, PM treatment significantly improved MG-induced deterioration of osteoblastic differentiation in MC3T3 cells (Figure 4). In this assay, we used MGO at concentrations of 0.5 to 2.0 mM equivalent to those in our previous study [11]. The concentration of MGO in vivo remains controversial, and the data vary among multiple assay methods. The actual intracellular concentration of MGO has been argued in living organs and cells. In addition, the concentrations of PM used for in vitro cell-based experiments are still under discussion along with MGO doses. Pyridoxine, a form of vitamin B6, has been reported to cause cell death at 1 μM [23]. In contrast, pyridoxal and PM are known to be nontoxic. No or minimal adverse events of PM have been observed in previous clinical studies.

DM can cause many complications [24]. The mechanism of diabetes-associated bone problems has been assumed that bone marrow-derived mesenchymal stem cells would not be recruited to the injured site in diabetic patients [25], and therefore, diabetes has been related to osteoblast suppression and osteoclast-promoting actions [24]. Our previous study demonstrated that glycation stress, including MGO, could impair osteoblastic differentiation and delay bone injury repair in diabetes [11]. In this study, PM administration rescued bone injury repair in mice with diabetes. Thus, we propose that inhibition of MGO production could be a potentially useful strategy against bone problems in diabetes and PM could be a powerful candidate for MGO detoxification.

PM, a derivative of vitamin B6, is reported to have many biological effects: (1) inhibition of AGE formation by trapping dicarbonyl intermediates, including MGO, during the glycation reaction, (2) scavenging of toxic carbonyl products of glucose and lipid degradation, and (3) scavenging of reactive oxygen species (ROS) [12]. Oral PM treatment is reported to have benefits related to creatinine clearance and level of urinary transforming growth factor-β 1 in patients with type 1 and type 2 diabetes [26]. A recent study demonstrated the inhibitory effects of short- and long-term treatment of PM on the adhesive function of neutrophils and platelets in the microvessels of sickle cell disease mice [27], suggesting a novel biological action of PM.

In conclusion, our findings suggest that PM treatment could be useful in reducing glycative stress and improving bone union associated with diabetes. This is the first report on the beneficial effects of PM in bone injury and provides a basis for the treatment of pseudoarthrosis and delayed bone healing/fusion in patients with diabetes.

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Declaration of competing interest

The authors declare no conflicts of interest to report.

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