

# Cilostazol ameliorates high glucose-induced osteoblast dysfunction by modulating mitogen-activated protein kinase signaling and the OPG/RANKL axis

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**Abstract.** Diabetic osteopathy, characterized by impaired bone formation and increased fracture risk, is a complication of diabetes mellitus, with chronic hyperglycemia-induced oxidative stress contributing to osteoblast dysfunction. Cilostazol (CLZ) is a selective phosphodiesterase 3 inhibitor; however, its effects on bone cell function under metabolic stress remain unclear. The present study investigated the effects of CLZ on osteoblast function and the mitogen-activated protein kinase (MAPK) signaling network under oxidative stress induced by high glucose (HG) and glucose oxidase (GOx) in MC3T3-E1 osteoblastic cells. Osteogenic marker expression and MAPK signaling were evaluated by western blotting, while osteoblast differentiation was assessed using alkaline phosphatase and Alizarin Red S assays. Exposure to 50 mM HG and 5 mU/ml GOx markedly suppressed key osteogenic markers, including bone morphogenetic protein 2 and 7, Runt-related transcription factor 2, Osterix and collagen type I  $\alpha 1$  chain, decreased osteoprotegerin (OPG) expression and increased receptor activator of NF- $\kappa$ B ligand (RANKL) expression. However, CLZ (0.1 and 1  $\mu$ M) restored the expression of these osteogenic factors, increased OPG expression, decreased RANKL expression and restored alkaline phosphatase activity and mineralized nodule formation. CLZ treatment was associated with increased p38 and ERK phosphorylation and decreased JNK phosphorylation in HG/GOx-treated cells. These findings suggest that CLZ may attenuate HG/GOx-induced osteoblast

dysfunction through modulation of MAPK signaling and the OPG/RANKL axis. However, further *in vivo* studies are required to determine its translational relevance in diabetic bone healing.

## Introduction

Diabetes mellitus is a chronic metabolic disorder defined by persistent hyperglycemia, leading to various systemic complications, including diabetic osteopathy (1). This condition encompasses a broad range of skeletal alterations, including impaired bone mineral density, deteriorated bone quality and suppressed bone turnover, collectively resulting in compromised mechanical integrity (2). While both type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) increase skeletal fragility, their underlying clinical profiles differ: T1DM is typically associated with reduced bone mass, whereas T2DM often presents with normal or even elevated bone mineral density, yet with markedly diminished bone quality, including alterations in bone microarchitecture and increased cortical porosity (2,3). Consequently, patients with diabetes have a higher risk of bone fractures, particularly hip fractures, which are 1.5-3 times more common than in normal controls, and exhibit delayed bone healing (4,5).

A previous epidemiological study suggested that the duration of diabetes and poor glycemic control independently increase skeletal fragility risk (6). This is partly attributable to the accumulation of advanced glycation end-products, which impair bone matrix properties and increase oxidative stress (7,8). Chronic high glucose (HG) exposure promotes excessive reactive oxygen species (ROS) production, causing oxidative damage in the bone microenvironment (9). This oxidative stress drives pathological disruption of bone remodeling and inhibits key osteogenic differentiation factor expression (10).

Osteoblast differentiation involves a network of signaling pathways and transcription factors. Bone morphogenetic proteins (BMPs), particularly BMP-2 and BMP-7, initiate osteogenesis, while Runt-related transcription factor 2 (RUNX2) and Osterix regulate osteoblast maturation and extracellular matrix synthesis (11,12). In addition, osteoprotegerin (OPG) and receptor activator of NF- $\kappa$ B ligand (RANKL)

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**Abbreviations:** OPG, osteoprotegerin; RANKL, receptor activator of NF- $\kappa$ B ligand; HG, high glucose; GOx, glucose oxidase

**Key words:** cilostazol, diabetes mellitus, MAPK signaling pathway, osteogenesis, oxidative stress

are key regulators of bone remodeling, with OPG acting as a decoy receptor that inhibits RANKL-mediated osteoclast differentiation. Furthermore, mitogen-activated protein kinase (MAPK) signaling pathways, including p38, ERK1/2 and JNK, are involved in mediating osteogenic signals (13). While p38 and ERK activation generally promote bone formation, oxidative stress-induced JNK pathway activation antagonizes BMP signaling and inhibits osteoblastic activity (14,15).

Cilostazol (CLZ), a selective phosphodiesterase 3 inhibitor, is clinically approved for treating intermittent claudication and peripheral vascular disease (16). Beyond its antiplatelet and vasodilatory effects, studies have reported that CLZ increases intracellular cyclic adenosine monophosphate (cAMP) levels and exerts protective effects in a number of tissue types, including promotion of bone growth and protection of hepatocytes against ethanol-induced oxidative stress and apoptosis (17,18). Previous studies have reported that CLZ exerts protective effects in synovial cells and vascular endothelial cells through activation of nuclear factor erythroid 2-related factor 2 and sirtuin 1 signaling pathways, respectively (19,20). However, the specific mechanisms through which CLZ modulates osteoblast function under HG-induced conditions remain unclear.

To investigate these mechanisms under diabetic-like conditions, MC3T3-E1 cells were exposed to a combination of HG and glucose oxidase (GOx). HG was used to simulate chronic hyperglycemia, whereas GOx was used to continuously generate hydrogen peroxide, thereby enhancing oxidative stress. This experimental model was employed to reproduce the combined hyperglycemic and oxidative stress conditions associated with diabetic osteoblast dysfunction (10,21,22). The present study aimed to investigate the protective effects of CLZ on osteoblast dysfunction under diabetic-like conditions, with particular focus on MAPK signaling and the OPG/RANKL axis. These findings are expected to provide mechanistic insights into the effects of CLZ on osteoblast function under diabetic-like conditions and serve as a basis for future *in vivo* and translational studies.

## Materials and methods

**Cell culture and reagents.** MC3T3-E1 subclone 4, a mouse osteoblastic cell line, was obtained from the American Type Culture Collection. Cells were cultured in  $\alpha$ -minimum essential medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. CLZ (Sigma-Aldrich; Merck KGaA) was dissolved in DMSO to prepare a 100 mM stock solution. For the cell viability assay, control cells received complete culture medium without CLZ or DMSO. For HG/GOx experiments, untreated control cells received the corresponding culture medium without HG, GOx, CLZ or DMSO. The final DMSO concentration in the CLZ-treated groups was  $\leq 0.001\%$  in the main experiments and  $\leq 0.01\%$  in the cell viability assay. Cells were treated with 50 mM HG and 5 mU/ml GOx (both from Sigma-Aldrich; Merck KGaA) to induce oxidative stress.

**Cell viability assay.** Cell viability was evaluated using a Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Inc.). MC3T3-E1 cells were seeded in 96-well plates at a density of

$1 \times 10^4$  cells/well and treated with various concentrations of CLZ (0.1, 1, 5 and 10  $\mu$ M) for 24 and 48 h at 37°C. After incubation, CCK-8 solution was added to each well, followed by incubation for 2 h at 37°C and absorbance at 450 nm was measured using a microplate reader. Cell morphology was observed using an inverted light microscope after treatment with CLZ (0, 0.1, 1, 5 or 10  $\mu$ M) for 24 and 48 h as aforementioned.

**Osteogenic differentiation.** MC3T3-E1 cells were seeded at a density of  $2 \times 10^4$  cells/cm<sup>2</sup> in either 24-well plates for ALP staining and activity assays or 12-well plates for Alizarin Red S (ARS) staining. For osteogenic differentiation assays, MC3T3-E1 cells were first exposed to HG (50 mM) and GOx (5 mU/ml) in complete culture medium for 4 h at 37°C. After exposure, the medium was replaced with osteogenic medium containing 50  $\mu$ g/ml L-ascorbic acid and 10 mM  $\beta$ -glycerophosphate to induce osteoblast differentiation, and either no CLZ or CLZ (0.1 or 1  $\mu$ M). The medium was replaced every 2-3 days while maintaining the respective treatments.

On day 7, ALP staining was performed using an Alkaline Phosphatase Stem Cell Staining Kit (cat. no. SCR004; Sigma-Aldrich; Merck KGaA) according to the manufacturer's instructions. Cells were incubated with the staining reagent for 15 min at room temperature, and stained cells were observed using an inverted light microscope. ALP activity was measured using a TRACP & ALP Assay Kit (cat. no. MK301; Takara Bio Inc.). The assay reaction was performed for 30 min at 37°C and absorbance was measured at 405 nm using a microplate reader. ALP activity was normalized to the total protein amount determined using a BCA assay. On day 14, cells were fixed with 10% neutral buffered formalin for 30 min at room temperature and stained with 2% ARS for 30 min at room temperature in the dark. Stained mineralized nodules were visualized using a light microscope. For quantification, bound ARS was solubilized with 10% cetylpyridinium chloride for 30 min at room temperature. The extracted solution was transferred to a 96-well plate and absorbance was measured at 562 nm using a microplate reader.

**Western blot analysis.** MC3T3-E1 cells were first exposed to HG (50 mM) and GOx (5 mU/ml) simultaneously for 4 h at 37°C to induce oxidative stress. After stimulation, the medium was replaced with either normal growth medium or normal growth medium containing CLZ (0.1 or 1  $\mu$ M). Cells were subsequently cultured for different durations depending on the experimental purpose. For western blot analysis of osteogenic markers, protein lysates were collected after an additional 48-h incubation. For MAPK signaling analysis, protein lysates were collected after an additional 24-h incubation.

Subsequently, proteins were extracted using RIPA buffer (Thermo Fisher Scientific, Inc.) and protein concentrations were determined using a BCA assay. Equal amounts of protein (10  $\mu$ g/lane) were separated by 10 or 12% SDS-PAGE, depending on the molecular weight of the target protein, before being transferred onto PVDF membranes. The membranes were blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature and incubated overnight at 4°C with the following primary antibodies: BMP-2 (cat. no. PA5-85956; 1:2,000; Thermo Fisher Scientific, Inc.),

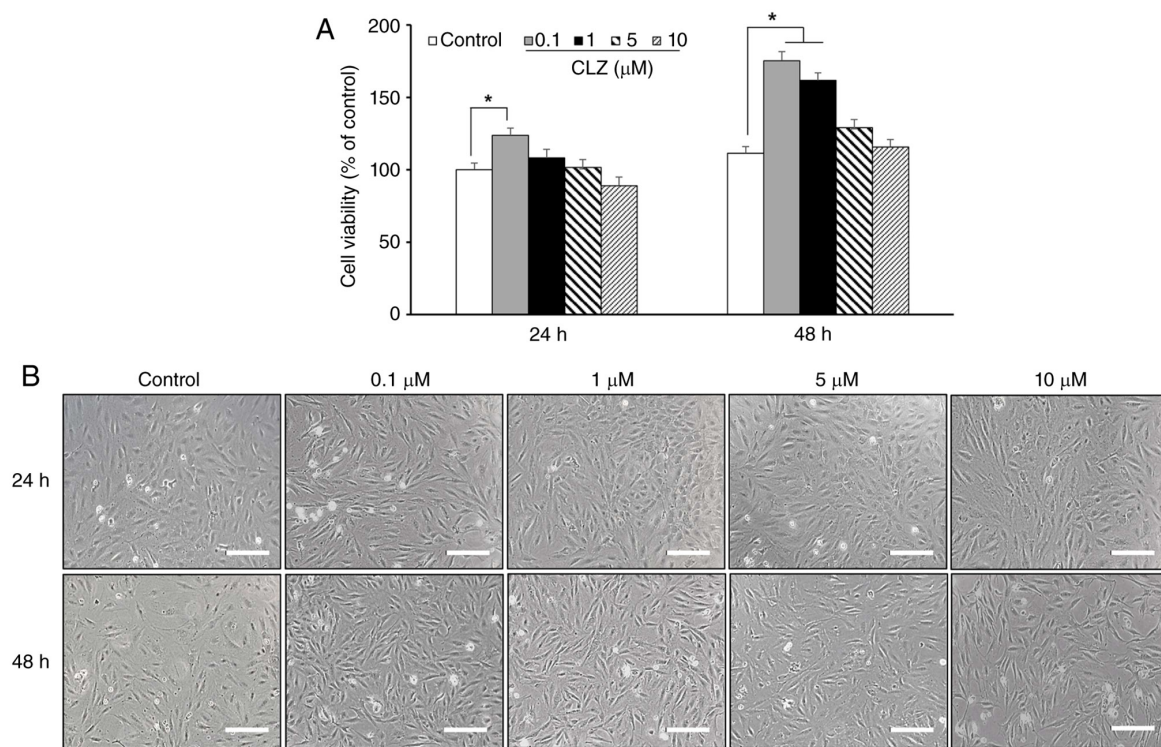


Figure 1. Effects of CLZ on MC3T3-E1 cell viability and morphology. (A) Cells were treated with CLZ (0.1-10  $\mu$ M) for 24 and 48 h, and viability was assessed using the Cell Counting Kit-8 assay. (B) Representative images of cell morphology after 24 and 48 h of CLZ treatment (scale bar, 100  $\mu$ m). Data are presented as the mean  $\pm$  SD of three independent experiments. \* $P$ <0.05. CLZ, cilostazol.

BMP-7 (cat. no. BS3674; 1:2,000; Bioworld Technology, Inc.), RUNX2 (clone D1L7F; cat. no. 12556; 1:2,000; Cell Signaling Technology, Inc.), Osterix (cat. no. ab22552; 1:1,000; Abcam), collagen type I  $\alpha$ 1 chain (COL1A1; cat. no. BS70155; 1:2,000; Bioworld Technology, Inc.), OPG (cat. no. BS6684; 1:2,000; Bioworld Technology, Inc.), RANKL (cat. no. BS72037; 1:2,000; Bioworld Technology, Inc.), phosphorylated (p)-p38 MAPK (Thr180/Tyr182; cat. no. 4511; 1:2,000; Cell Signaling Technology, Inc.), p38 MAPK (clone D13E1; cat. no. 8690; 1:2,000; Cell Signaling Technology, Inc.), p-p44/42 MAPK (ERK1/2) (Thr202/Tyr204; clone 197G2; cat. no. 4377; 1:2,000; Cell Signaling Technology, Inc.), p44/42 MAPK (ERK1/2) (clone 137F5; cat. no. 4695; 1:2,000; Cell Signaling Technology, Inc.), p-SAPK/JNK (Thr183/Tyr185; cat. no. 4668; 1:2,000; Cell Signaling Technology, Inc.), SAPK/JNK (cat. no. 9252; 1:2,000; Cell Signaling Technology, Inc.) and  $\beta$ -actin (I102; cat. no. AP0060; 1:5,000; Bioworld Technology, Inc.). After washing three times with TBST, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (cat. no. 7074; 1:2,000; Cell Signaling Technology, Inc.) for 1 h at room temperature. Protein bands were visualized using Amersham ECL reagent (Cytiva) and semi-quantified using ImageJ software (version 1.53v; National Institutes of Health). Depending on the molecular weights of the target proteins, the membranes were either stripped and re-probed sequentially or separate membranes were used for individual target proteins.  $\beta$ -actin was used as a loading control on each membrane. The target proteins are presented together with their respective  $\beta$ -actin loading control from the same membrane in each figure.

**Statistical analysis.** Data are presented as the mean  $\pm$  SD of three independent biological replicates. For the CCK-8, ALP and ARS assays, each experimental condition was analyzed in triplicate wells within each independent biological experiment. Western blot analysis was performed using samples from three independent biological experiments. Data normality was assessed using the Shapiro-Wilk test. Statistical analyses were performed using GraphPad Prism version 9.0 (Dotmatics). Comparisons among multiple groups were performed using one-way ANOVA followed by Tukey's post hoc test.  $P$ <0.05 was considered to indicate a statistically significant difference.

## Results

**CLZ exhibits no cytotoxicity and maintains cellular morphology in MC3T3-E1 cells.** MC3T3-E1 cells were treated with various concentrations of CLZ (0.1, 1, 5 and 10  $\mu$ M) for 24 and 48 h to assess its potential cytotoxicity in osteoblasts. CCK-8 assay results showed that CLZ did not reduce cell viability at any of the tested concentrations or time points; however, cell viability was significantly increased following treatment with 0.1  $\mu$ M CLZ at both 24 and 48 h. This indicated high biocompatibility of CLZ with osteoblasts (Fig. 1A). Subsequently, the effect of CLZ treatment on cell morphology was examined. Morphological analysis using inverted microscopy revealed that CLZ-treated cells retained their typical spindle-shaped fibroblastic appearance, similar to that of the control group (Fig. 1B). These findings indicate that CLZ exhibited no detectable cytotoxicity within the tested concentration range. Therefore, 0.1 and 1  $\mu$ M were selected for subsequent experiments because these concentrations showed

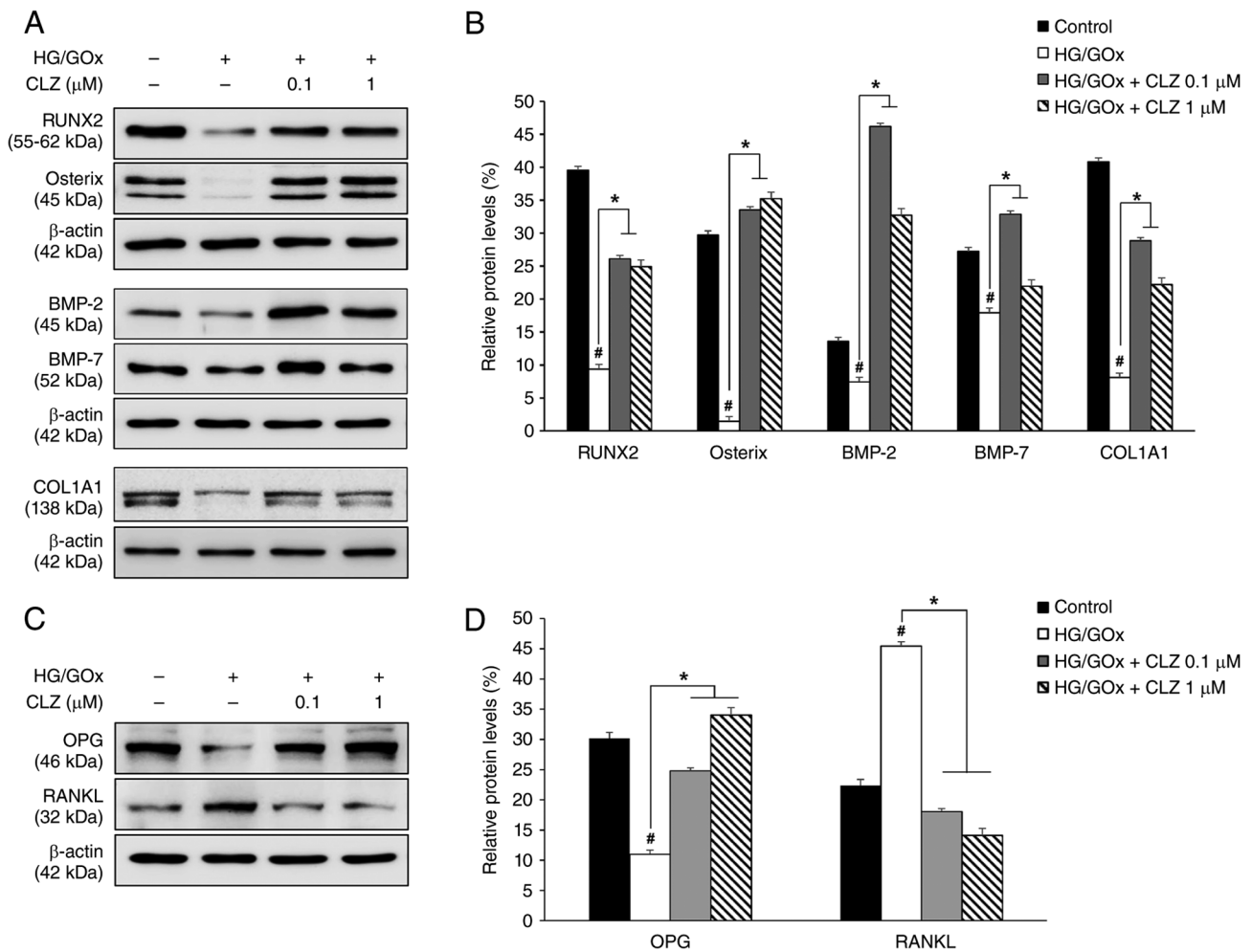


Figure 2. Effects of CLZ on osteogenic marker, OPG and RANKL expression under HG/GOx-induced stress. MC3T3-E1 cells were exposed to HG (50 mM) and GOx (5 mU/ml) for 4 h. After stimulation, the medium was replaced with either normal medium or medium containing CLZ (0.1 or 1  $\mu$ M), followed by incubation for an additional 48 h before protein extraction. (A) Representative western blot analysis of osteogenic markers (RUNX2, Osterix, BMP-2, BMP-7 and COL1A1). (B) Semi-quantitative densitometric analysis of osteogenic markers, normalized to  $\beta$ -actin. (C) Western blot analysis of OPG and RANKL protein levels. (D) Semi-quantitative densitometric analysis of OPG and RANKL protein expression, normalized to  $\beta$ -actin. Data are presented as the mean  $\pm$  SD of three independent experiments. \* $P$ <0.05 Control vs. HG/GOx groups and \* $P$ <0.05 HG/GOx vs. HG/GOx + CLZ groups. BMP, bone morphogenetic protein; CLZ, cilostazol; COL1A1, collagen type I  $\alpha$ 1 chain; GOx, glucose oxidase; HG, high glucose; OPG, osteoprotegerin; RANKL, receptor activator of NF- $\kappa$ B ligand; RUNX2, Runt-related transcription factor 2.

no evidence of cytotoxicity and were supported by previous *in vitro* studies demonstrating their biological activity (23,24).

**CLZ restores HG/GOx-induced downregulation of osteogenic markers and OPG/RANKL expression.** To evaluate the effect of CLZ on osteoblast differentiation and bone remodeling balance under diabetic-like conditions, the expression of key proteins was measured. Western blot analysis revealed that HG/GOx exposure markedly reduced the protein levels of RUNX2, Osterix, BMP-2, BMP-7 and COL1A1 (Fig. 2A). Semi-quantitative densitometric analysis indicated that CLZ (0.1 and 1  $\mu$ M) significantly increased the expression of these osteogenic markers following HG/GOx-induced oxidative stress (Fig. 2B). Subsequently, the expression of OPG and RANKL was assessed. HG/GOx treatment decreased OPG expression and increased RANKL expression (Fig. 2C). However, CLZ treatment increased OPG expression and reduced RANKL expression following HG/GOx exposure (Fig. 2D). These findings suggest that CLZ may contribute

to the restoration of osteoblast differentiation and the OPG/RANKL balance under HG-induced stress.

**CLZ modulates HG/GOx-induced MAPK signaling dysregulation.** To investigate the association between CLZ treatment and MAPK signaling, MAPK phosphorylation was assessed. Western blot analysis revealed that HG/GOx treatment markedly decreased p38 and ERK phosphorylation and increased JNK phosphorylation (Fig. 3A). Semi-quantitative densitometric analysis of the phosphorylated-to-total protein ratios showed that HG/GOx treatment decreased the p-ERK/ERK and p-p38/p38 ratios and increased the p-JNK/JNK ratio compared with the Control group, whereas CLZ treatment partially reversed these changes (Fig. 3B). CLZ also attenuated the HG/GOx-induced increase in p-JNK levels (Fig. 3B). These findings suggest that CLZ-mediated modulation of MAPK phosphorylation, characterized by increased p38 and ERK phosphorylation and decreased JNK phosphorylation, may

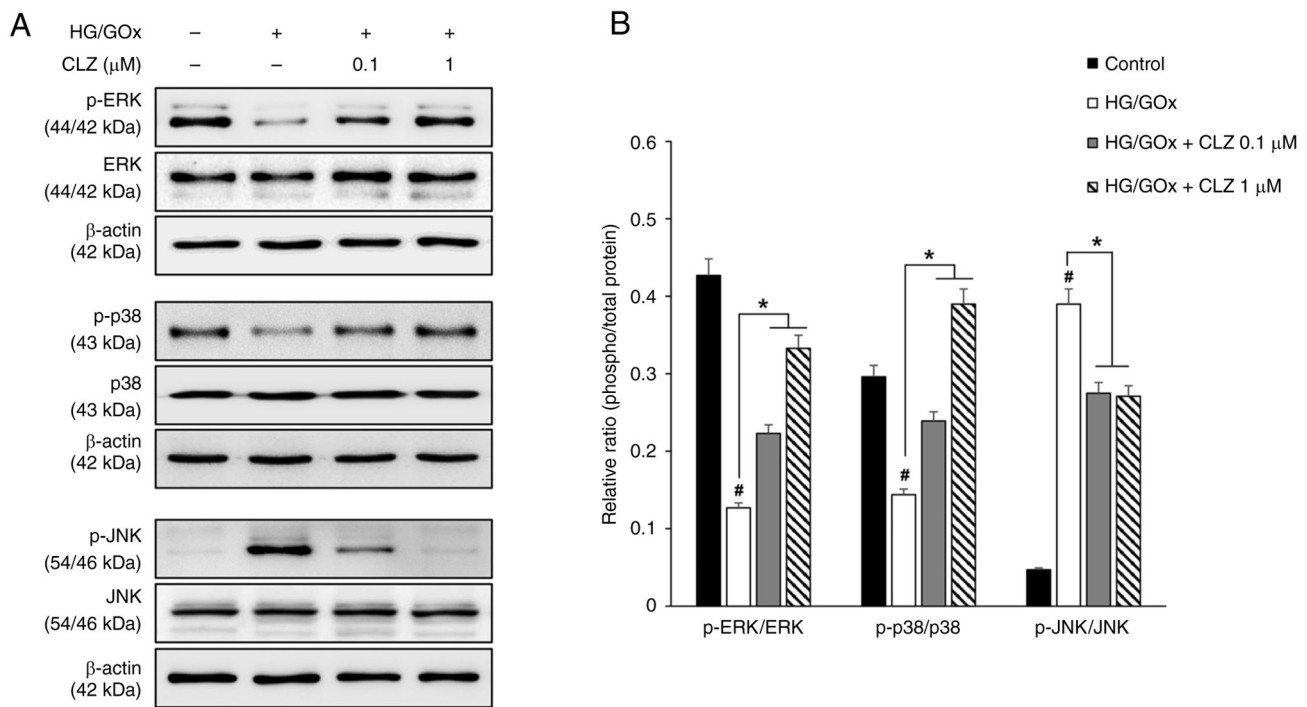


Figure 3. CLZ modulates mitogen-activated protein kinase phosphorylation under HG/GOx-induced stress. MC3T3-E1 cells were exposed to HG (50 mM) and GOx (5 mU/ml) for 4 h. After stimulation, the medium was replaced with either normal medium or medium containing CLZ (0.1 or 1 μM), followed by incubation for an additional 24 h before protein extraction and western blot analysis. (A) Representative western blot analysis of p- and total forms of p38, JNK and ERK; β-actin served as a loading control. (B) Semi-quantitative densitometric analysis of MAPK phosphorylation, expressed as the ratios of p-ERK/ERK, p-p38/p38 and p-JNK/JNK. Data are presented as the mean ± SD of three independent experiments. <sup>#</sup>P<0.05 Control vs. HG/Gox groups and <sup>\*</sup>P<0.05 HG/Gox vs. HG/Gox + CLZ groups. CLZ, cilostazol; GOx, glucose oxidase; HG, high glucose; p-, phosphorylated.

contribute to restoring signaling conditions favorable for osteoblast maturation under diabetic-like conditions.

**CLZ restores HG/GOx-induced suppression of ALP activity and mineralized nodule formation.** To assess the functional effect of CLZ on osteoblast differentiation, ALP activity and calcium deposition were assessed. HG/GOx treatment markedly reduced ALP staining (Fig. 4A) and activity (Fig. 4B). However, treatment with CLZ (0.1 and 1 μM) significantly restored ALP activity (Fig. 4B). Subsequently, ARS staining was performed to assess late-stage osteogenesis by evaluating mineralized nodule formation. HG/GOx exposure markedly reduced calcium-rich deposits, whereas CLZ treatment increased mineralized nodule formation (Fig. 4C). Quantitative analysis of the ARS staining also indicated significantly increased mineralization following CLZ treatment in HG/GOx-exposed cells (Fig. 4D). Overall, these findings suggest that CLZ may restore the osteogenic potential of MC3T3-E1 cells under diabetic-like conditions.

**Schematic model of the protective effect of CLZ against HG/GOx-induced osteogenic dysfunction.** Based on the aforementioned findings, a schematic model summarizing the proposed effects of CLZ on osteoblast dysfunction under diabetic-like conditions is presented in Fig. 5. HG/GOx treatment was associated with decreased p38 and ERK phosphorylation and increased JNK phosphorylation. These changes were accompanied by decreased expression of key osteogenic factors, including RUNX2, Osterix, BMP-2, BMP-7 and COL1A1, decreased OPG expression, increased

RANKL expression, reduced ALP activity and decreased mineralized nodule formation. CLZ treatment was associated with partial restoration of these changes, including altered MAPK phosphorylation, increased osteogenic marker expression and improved osteogenic function in MC3T3-E1 cells.

## Discussion

The effects of CLZ on osteoblast dysfunction and the potential underlying molecular mechanisms were investigated in MC3T3-E1 cells under HG/GOx-induced diabetic-like conditions. Excessive ROS production contributes to osteoblast dysfunction and impaired bone formation in diabetic osteopathy (25). In the present study, CLZ restored the expression of osteogenic markers suppressed under HG/GOx-induced stress, suggesting that CLZ may contribute to the recovery of osteoblast function under diabetic-like conditions. CLZ increased the expression of key osteogenic markers, including BMP-2 and BMP-7, along with the master transcription factors RUNX2 and Osterix. BMP-2 and BMP-7 trigger osteogenesis by activating Smad-dependent and -independent signaling pathways, directly enhancing RUNX2 transcriptional activity (26). Furthermore, the recovery of RUNX2 and Osterix, which regulate downstream genes including ALP and COL1A1, suggests that CLZ may support osteoblastic differentiation (12). The simultaneous increase in these factors indicates that CLZ may contribute to the restoration of the molecular network involved in osteoblast differentiation under diabetic-like conditions.

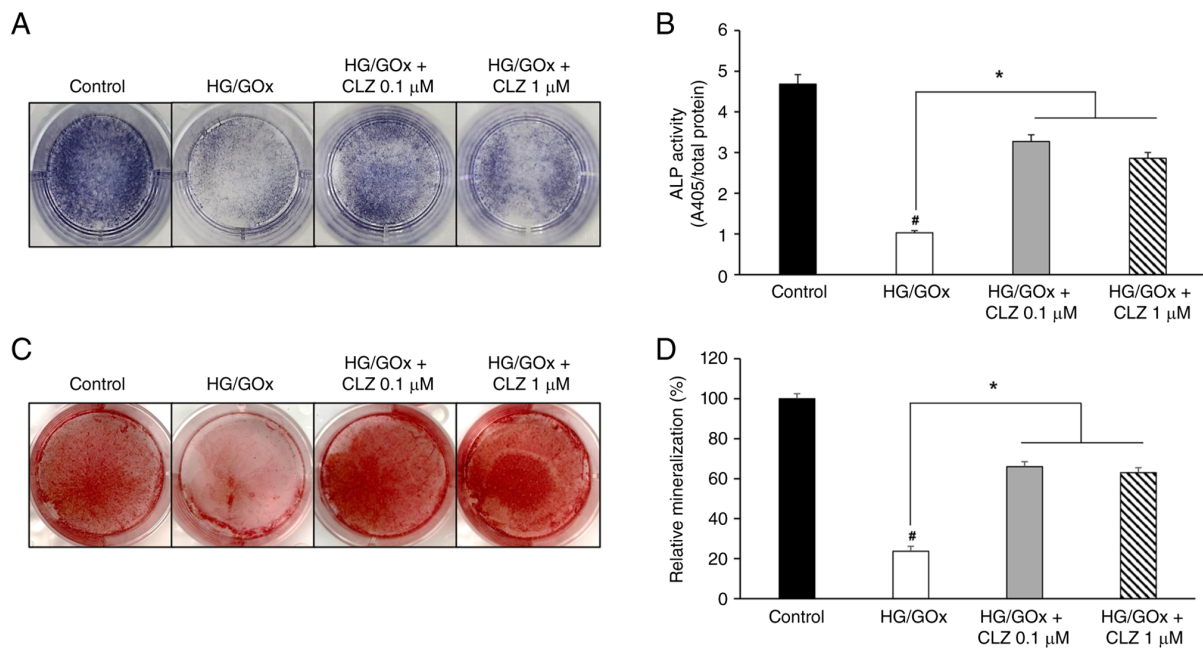


Figure 4. CLZ restores ALP activity and mineralized nodule formation under HG/GOx-induced stress. MC3T3-E1 cells were first exposed to HG (50 mM) and GOx (5 mU/ml) for 4 h. After exposure, the medium was replaced with osteogenic induction medium containing either no CLZ or CLZ (0.1 or 1  $\mu$ M). Cells were maintained under these conditions throughout the differentiation period, and the treatment medium was replaced every 2-3 days. (A) Representative images of ALP staining on day 7. (B) Quantitative analysis of ALP activity measured at A405 and normalized to the total protein amount. (C) Representative ARS-stained images on day 14 showing mineralized nodules. (D) Quantitative analysis of ARS staining. Data are presented as the mean  $\pm$  SD of three independent experiments. <sup>#</sup> $P < 0.05$  Control vs. HG/GOx groups and <sup>\*</sup> $P < 0.05$  HG/GOx vs. HG/GOx + CLZ groups. ALP, alkaline phosphatase; ARS, Alizarin Red S; CLZ, cilostazol; GOx, glucose oxidase; HG, high glucose; A405, absorbance at 405 nm.

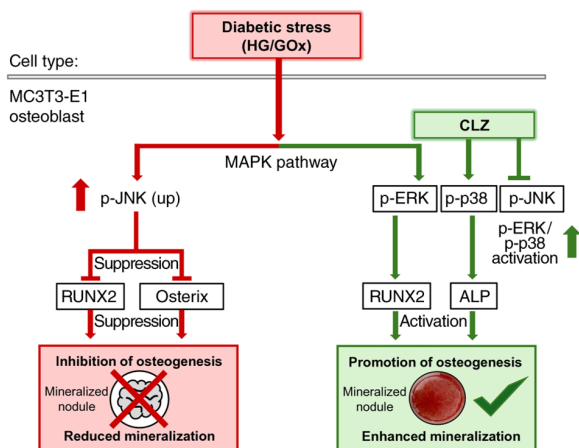


Figure 5. Schematic illustration of the effects of CLZ on osteogenesis under HG/GOx-induced oxidative stress. HG/GOx disrupts MAPK signaling by suppressing p-p38 and p-ERK while activating p-JNK, accompanied by suppression of RUNX2 and Osterix and reduced mineralization. CLZ modulates MAPK signaling, accompanied by restoration of RUNX2 expression, ALP activity and mineralization in MC3T3-E1 cells. This schematic summarizes the proposed mechanism based on the findings of the present study. Solid arrows indicate activation and T-shaped lines indicate inhibition. Red indicates HG/GOx-induced detrimental changes, whereas green indicates CLZ-associated restorative changes. ALP, alkaline phosphatase; CLZ, cilostazol; GOx, glucose oxidase; HG, high glucose; MAPK, mitogen-activated protein kinase; p-, phosphorylated; RUNX2, Runt-related transcription factor 2.

The present findings suggested that the protective effects of CLZ are associated with modulation of MAPK signaling pathways. Previous studies have shown that p38 MAPK

is essential for late osteoblast maturation, while ERK1/2 signaling regulates early osteoblast commitment and proliferation (13,14). By contrast, excessive JNK activation under oxidative stress has been reported to be associated with osteoblast dysfunction and apoptosis (15). In the present study, CLZ restored p38 and ERK phosphorylation while attenuating JNK activation, suggesting that modulation of MAPK signaling may contribute to the restoration of osteogenic function under diabetic-like conditions. However, the precise upstream mechanism by which CLZ regulates MAPK phosphorylation remains to be fully elucidated. It has been demonstrated that CLZ exerts antioxidant and anti-inflammatory effects through phosphodiesterase 3 inhibition and the subsequent elevation of intracellular cAMP levels (27). These protective actions, including the suppression of mitochondria-dependent apoptotic signaling and activation of ERK1/2 and p38 MAPK pathways in endothelial cells, have been previously reported and may be associated with the MAPK signaling changes observed in the present study (28). Such modulation of MAPK signaling, together with the attenuation of JNK activation observed in the present study, may ultimately facilitate the recovery of osteogenic gene expression and osteoblast function under diabetic-like conditions.

Moreover, the OPG/RANKL axis is important in regulating the bone formation-resorption imbalance in diabetic osteopathy (29). A low OPG/RANKL ratio, a hallmark of bone loss in patients with diabetes, often promotes osteoclastogenesis (30). By increasing OPG expression and decreasing RANKL expression, CLZ may support osteoblast function and contribute to the regulation of bone remodeling under diabetic-like conditions.

Despite these results, several limitations should be acknowledged. First, although changes in MAPK phosphorylation were observed, the present study did not directly evaluate intracellular oxidative stress using assays measuring ROS, superoxide dismutase, malondialdehyde and glutathione peroxidase levels. Therefore, further studies incorporating these oxidative stress-related analyses, as well as specific MAPK inhibitors or activators, are required to determine whether CLZ directly modulates MAPK signaling or exerts its protective effects indirectly through the attenuation of oxidative stress. Second, although JNK signaling has been implicated in oxidative stress-induced apoptosis, the present study did not directly assess osteoblast apoptosis using apoptosis-specific assays, such as TUNEL staining, Annexin V-FITC flow cytometry or the expression of apoptosis-related proteins, including Bax, Bcl-2 and cleaved caspase-3. Therefore, whether CLZ influences JNK-mediated apoptosis remains to be determined through additional apoptosis-specific assays. Third, although CLZ increased OPG expression and decreased RANKL expression, the present study did not directly evaluate osteoclast differentiation or function. Therefore, additional studies using tartrate-resistant acid phosphatase staining, osteoclast functional assays or osteoblast-osteoclast co-culture models are warranted to determine whether these molecular changes translate into functional regulation of bone remodeling. Finally, the present study was conducted using a single osteoblastic cell line (MC3T3-E1), which limits the direct clinical translation of the findings. Therefore, further validation in diabetic animal models and clinical studies is required to determine the potential relevance of CLZ *in vivo*.

In conclusion, CLZ may ameliorate HG/GOx-induced osteoblast dysfunction by modulating MAPK phosphorylation and altering OPG and RANKL expression under diabetic-like conditions. These findings provide insight into the effects of CLZ on osteoblast function under diabetic-like conditions and set a basis for future *in vivo* studies.

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### Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

### Authors' contributions

JEK conceived and designed the study, performed the experiments, analyzed and interpreted the data and drafted the manuscript. HP supervised the study, contributed to data

interpretation and critically revised the manuscript for important intellectual content. JEK and HP confirm the authenticity of all the raw data. Both authors read and approved the final manuscript and agree to be accountable for all aspects of the work.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

### Use of artificial intelligence tools

During the preparation of this work, artificial intelligence tools (GPT-5.5; OpenAI) were used to improve the readability and language of the manuscript, and subsequently, the authors revised and edited the content produced by the artificial intelligence tools as necessary, taking full responsibility for the ultimate content of the present manuscript.

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