N,N-Dimethylformamide inhibits high glucose-induced osteoporosis via attenuating MAPK and NF-κB signalling

Aims
The role of N,N-dimethylformamide (DMF) in diabetes-induced osteoporosis (DM-OS) progression remains unclear. Here, we aimed to explore the effect of DMF on DM-OS development.

Methods
Diabetic models of mice, RAW 264.7 cells, and bone marrow macrophages (BMMs) were established by streptozotocin stimulation, high glucose treatment, and receptor activator of nuclear factor-κB ligand (RANKL) treatment, respectively. The effects of DMF on DM-OS development in these models were examined by micro-CT analysis, haematoxylin and eosin (H&E) staining, osteoclast differentiation of RAW 264.7 cells and BMMs, H&E and tartrate-resistant acid phosphatase (TRAP) staining, enzyme-linked immunosorbent assay (ELISA) of TRAP5b and c-terminal telopeptides of type 1 (CTX1) analyses, reactive oxygen species (ROS) analysis, quantitative reverse transcription polymerase chain reaction (qRT-PCR), Cell Counting Kit-8 (CCK-8) assay, and Western blot.

Results
The established diabetic mice were more sensitive to ovariectomy (OVX)-induced osteoporosis, and DMF treatment inhibited the sensitivity. OVX-treated diabetic mice exhibited higher TRAP5b and c-terminal telopeptides of type 1 (CTX1) levels, and DMF treatment inhibited the enhancement. DMF reduced RAW 264.7 cell viability. Glucose treatment enhanced the levels of TRAP5b, cathepsin K, Atp6v0d2, and H+-ATPase, ROS, while DMF reversed this phenotype. The glucose-increased protein levels were inhibited by DMF in cells treated with RANKL. The expression levels of antioxidant enzymes Gclc, Gclm, Ho-1, and Nqo1 were upregulated by DMF. DMF attenuated high glucose-caused osteoclast differentiation by targeting mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) signalling in BMMs.

Conclusion
DMF inhibits high glucose-induced osteoporosis by targeting MAPK and NF-κB signalling.

Keywords: DMF, Osteoporosis, Diabetes

Article focus
This study aimed to explore the effects of N,N-dimethylformamide (DMF) on diabetes-induced osteoporosis (DM-OS) development.

Key messages
Streptozotocin-stimulated diabetic mice were more sensitive to ovariectomy (OVX)-induced osteoporosis, and DMF treatment significantly inhibited the osteoporosis.

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DMF treatment attenuated high glucose-caused osteoclast differentiation by targeting mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) signalling in bone marrow macrophages.

Strengths and limitations
- These results demonstrate that DMF inhibits high glucose-induced osteoporosis by targeting MAPK and NF-κB signalling.
- The effects of DMF treatment on DM-OS need to be further confirmed in clinical trials.

Introduction
Osteoporosis is a disorder that reduces bone strength and increases the risk of bone fracture, and the most prevalent basis of bone fractures for older patients. Increasing investigations have revealed that about half of the female and one-quarter of the male population will suffer from osteoporosis-induced bone fractures. Because osteoporosis is associated with other diseases, bone loss has become a serious health problem worldwide. Diabetes mellitus (DM) is an essential risk factor contributing to the progress of osteoporosis. DM-induced osteoporosis (DM-OS) is a general metabolic bone disease that enhances microstructural changes in bone tissues and osteopenia-related fractures, increases friability, and reduces bone strength, a primary complication of DM impacting the skeletal system. With the morbidity of DM-OS dramatically increasing, DM-OS has become the principal cause of death and mutilation in diabetes patients, seriously affecting their quality of life and creating a substantial economic burden to their families and society. Therefore, there is an urgent need to understand the mechanisms underlying DM-OS.

N,N-dimethylformamide (DMF) is an efficient multipurpose polar catalyst for numerous chemical reactions. It can also be used as an efficient ligand in constructing metallic complexes, and as a dehydrating/reducing agent to participate in reactions. More importantly, due to its special structure, DMF is involved in many reactions by functioning as a multi-purpose building block of different units. The biomedical activity of DMF is well-identified in multiple diseases, such as inflammation and cancer. However, the effect of DMF on DM-OS remains unknown.

Mitogen-activated protein kinase (MAPK) signalling, an essential pathway in multiple physiological and pathological processes, plays a critical role in modulating osteoporosis, including DM-OS. It has been reported that high glucose suppresses osteogenic differentiation and promotes adipogenicity via cyclic adenosine 3’, 5’-monophosphate (cAMP)/protein-kinase A/extracellular signal-regulated kinase signalling in MG-63 cells. Activation of transcription factor-2 and P38 is involved in high extracellular glucose-related osteoblast osmotic response. Exosomes derived from bone marrow mesenchymal stem cells improve osteoporosis by increasing osteoblast proliferation via MAPK pathway. Reduced microRNA-182-5p levels assist alendronate to elevate osteoblast differentiation and proliferation in osteoporosis via Rap1/MAPK signalling pathway. Furthermore, nuclear factor kappa-B (NF-κB) signalling, a critical pathway in multiple cellular processes, contributes to osteoporosis development, and the receptor activator of NF-κB ligand (RANKL) is critically involved in bone healing. MLN64 knockdown inhibits diabetic osteoporosis and represses RANKL-stimulated osteoclastic differentiation by regulating NF-κB signalling in STZ-induced mice. Enhanced expression of the receptor of NF-κB activation decreases runt-related TF2 appearance in the bone from streptozocin-induced diabetic rats. Hydrogen gas prevents ovariectomy-related osteoporosis by repressing NF-κB signalling. Bilidobacterium longum NK49 and Lactobacillus Plantarum NK3 relieve bacterial vaginosis and osteoporosis by repressing NF-κB-related expression of tumour necrosis factor alpha (TNF-α). Glycyrrhizin acid inhibits postmenopausal osteoporosis and osteoclast differentiation by regulating c-jun N-terminal kinase (JNK), extracellular signal-related kinase (ERK), and NF-κB signalling. However, the effect of DMF on MAPK and NF-κB signalling in modulating DM-OS remains elusive.

In this study, we aimed to explore the role and the underlying mechanism of DMF in DM-OS progression, and identified a novel inhibitory effect of DMF on DM-OS by modulating MAPK and NF-κB signalling.

Methods
Animals. To investigate the effect of DMF on high glucose-induced osteoporosis, we established a glucose-related osteoporosis mouse model. In brief, 42 female C57BL/6 mice (20 to 22 g, ten weeks old) were randomly divided into control groups (n = 21) and streptozocin treatment groups (n = 21), which were further randomly assigned into sham group (n = 7), ovariectomy (OVX) group (n = 7), and OVX+DMF group (n = 7). Mice in the streptozocin group were intraperitoneally injected with 50 mg/kg streptozotocin (Sigma-Aldrich, USA) for five consecutive days. Blood glucose levels ≥ 16.7 mmol/l were considered as diabetes. Then, the impact of DMF on osteoporosis was analyzed in an OVX-induced osteoporosis mouse model, in which control and diabetic mice were ovariectomized and orally administered DMF (Sigma-Aldrich) daily (30 mg/kg) in 0.6% methocel emulsion (Sigma-Aldrich) for eight weeks. For OVX procedures, after anaesthesia, bilateral incisions were made, and the uterine horn just below the ovary was sutured, followed by ovary extraction. For sham mice, ovaries were raised through the incision site and lowered back. Finally, mice were euthanized, and the femora were obtained. Animal care and experimental procedures in this study were authorized by the Animal Ethics Committee.
of our institution. An ARRIVE checklist is included in the Supplementary Material to show that the ARRIVE guidelines were adhered to in this study.

**Micro-CT analysis.** Bone mineral density (BMD) of the femora was measured by dual-energy x-ray absorptiometry (Hologic Discovery DXA system, Hologic, USA), and a micro-CT system (Skyscan 1176; Bruker-MicroCT, Belgium) was used to analyze the bone microarchitecture and morphology. Femora were scanned at 80 keV and 309 μA with an aluminium plus copper filter, and the images were collected. The BMD, trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and bone volume per tissue volume (BV/TV) were analyzed using Skyscan CTAn v.1.1.7 software (Bruker-MicroCT).

**H&E and TRAP staining analysis of tissue samples.** Tissue samples were fixed in 4% paraformaldehyde at 4°C for 24 hours and decalcified in 20% ethylenediaminetetraacetic acid (EDTA) for two weeks. After that, the samples were dehydrated through an ethanol series, embedded in paraffin, and prepared as 4 μm thick sections using a microtome for haematoxylin and eosin (H&E) and tartrate-resistant acid phosphatase (TRAP) staining following the manufacturer’s instructions. Histomorphometric analyses of osteoclast numbers were performed using Image-Pro Plus (Media Cybernetics, USA).

**Cell culture and treatment.** BMMs were obtained from the bone marrow of eight-week-old C57BL/6 mice. The mice were euthanized to collect the tibiae and femora. BMMs were cultured in α-minimum essential medium (MEM) (Thermo Fisher Scientific, USA) with 10% fetal bovine serum and 1% penicillin/streptomycin. BMMs were plated into 24-well plates and treated with 10×10^3 BMMs were plated into 24-well plates and treated with 10% paraformaldehyde for 10 minutes and TRAP staining was performed using an acid phosphatase kit (Sigma-Aldrich). The dark red multinucleated cells were considered as TRAP-positive/nucleated cells.

**Quantitative reverse transcription polymerase chain reaction.** Total RNAs were extracted using TRIzOL (Thermo Fisher Scientific). The first-strand complementary DNA (cDNA) was synthesized using Stand cDNA Synthesis Kits (Thermo Fisher Scientific) following the manufacturer’s instructions. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was carried out using SYBR Real-time PCR kit (Takara, Japan) with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the endogenous control. Quantitative determination of RNA levels was conducted by SYBR GreenPremix Ex Taq II Kit (Takara). The experiments were repeated three times. The primers used for qPCR were H+-ATPase forward 5′-ATGGAGCGCTTCTTGATAAAGCAG-3′ and reverse: 5′-CAAGCCGATAGGAACCCGTA-3′; Atp6vod2 forward 5′-CTGGTCTGGAGATGCCAAAGC-3′ and reverse 5′-GTGGCATAGTCCGTTGCTG-3′; Cathepsin K forward 5′-AGGAAAGCAGACTAGGATA-3′ and reverse 5′-GCTGGCTGGAAATCACCTT-3′; Gclc forward 5′-CATACGGCTGATTCGACTGACC-3′ and reverse 5′-CCTCCATCCAGTAAAATGC-3′; Gclm forward 5′-AGGAGCTTGGGAGCTGACTCC-3′ and reverse 5′-GAGCCAGCCTTCAGCAGGACC-3′ and reverse 5′-CATCACCAGCCATGGAATTT-3′; Sqo1 forward 5′-AGGAGCGGGAGTACTGGAGAC-3′ and reverse 5′-TCCACCCACGATTGCTGTA-3′.

**TRAP5b and CTX1 detection.** After mice were euthanized, plasma samples were obtained. The expression levels of TRAP5b and CTX1 in plasma samples of mice were measured using enzyme-linked immunosorbent assay (ELISA) kits (TRAP5b and CTX1, Elabscience, China), and the absorbance at 450 nm was measured on a plate reader (BioTek EL 800, BioTek, USA). All procedures were conducted following the manufacturer’s instructions.

**Osteoclast differentiation and TRAP staining of cells.** Approximately 5×10^5 RAW 264.7 cells and 10×10^3 BMMs were plated into 24-well plates and treated with glucose and DMF at indicated doses under the treatment of RANKL (50 ng/ml, five days, R&D Systems, USA). Cells were then fixed in 10% paraformaldehyde for ten minutes and TRAP staining was performed using an acid phosphatase kit (Sigma-Aldrich). The dark red multinucleated cells were considered as TRAP-positive/nucleated cells.

**Intracellular reactive oxygen species measurement.** In brief, at various times after stimulation with RANKL, confluent cells were washed with α-MEM lacking penicillin G and incubated in the dark for five minutes in Krebs-Ringer solution containing 5 μM DCFH-DA. Cells were then observed under a fluorescent microscope and 2,7-dichlorofluorescein (DCF) fluorescence was measured at an excitation of 488 nm and emission of 515 to 540 nm. The fluorescence images were obtained with digital interference contrast, and the mean relative fluorescence intensity for each group of cells was measured using Image-Pro Plus.
N,N-dimethylformamide (DMF) inhibits high glucose-enhanced receptor activator of nuclear factor κB ligand (RANKL)-induced osteoclast differentiation in vitro. a) Cell counting kit-8 (CCK-8) measurement of the viability of RAW 264.7 cells treated with DMF at indicated concentrations. The RAW 264.7 cells were treated with glucose at the indicated doses. b) The representative images of tartrate-resistant acid phosphatase (TRAP) staining using an acid phosphatase kit (200×). c) The count of TRAP-positive multinucleated cells containing > three nuclei. d) The expression levels of cathepsin K, Atp6v0d2, and H+-ATPase analyzed by quantitative polymerase chain reaction (qPCR) assays. The RAW 264.7 cells were treated with glucose, DMF, or co-treated with glucose and DMF. e) The representative images of TRAP staining using an acid phosphatase kit (200×). f) The count of TRAP-positive multinucleated cells containing > three nuclei. g) The expression levels of cathepsin K, Atp6v0d2, and H+-ATPase measured by qPCR assays. h) and i) Resorption pits (200×) and j) and k) actin filament (F-ACTIN) were visualized and quantification in cells after seven days of treatment with receptor activator of nuclear factor κB ligand (RANKL) (50 ng/ml) and macrophage colony-stimulating factor (M-CSF) (30 ng/ml) (400×). Data were presented as mean and standard deviation of three independent experiments. *p < 0.05 versus control, #p < 0.05 versus glucose treatment (two-way analysis of variance). mRNA, messenger RNA.
CCK-8 assays. Cell viability was analyzed by cell counting kit-8 (CCK-8) assays. Approximately $5 \times 10^3$ cells were seeded into 96 wells. After having been cultured for 12 hours, cells were treated with DMF at different concentrations for 24 hours. The cells were then incubated with CCK-8 solution (KeyGEN Biotech, China) and cultured for another two hours at 37°C. Absorbance at 450 nm was measured using a microplate reader (BioTek EL 800).

Analysis of ROS levels. The intracellular ROS levels were measured by flow cytometry analysis. The glucose-treated N,N-dimethylformamide (DMF) inhibits high glucose-induced reactive oxygen species (ROS) accumulation. a) The glucose-treated (5 mM or 40 mM) bone marrow macrophages (BMMs) were preloaded with dichloro-dihydro-fluorescein diacetate (DCFH-DA) and treated with 50 ng/ml receptor activator of nuclear factor κB ligand (RANKL), 20 μM DMF, or their combination (+400). The ROS levels were assessed by flow cytometry analysis. The representative microscopic fields are shown: b) the relative intensity of DCF fluorescence in BMMs and d) RAW 264.7 cells. The c) glucose-treated (5 mM or 40 mM) BMMs and e) RAW 264.7 cells were treated with 50 ng/ml RANKL, 20 μM DMF, or a combination of the two. ROS levels were assessed by flow cytometry analysis. The glucose-treated (5 mM or 40 mM) RAW 264.7 cells were treated with DMF at the indicated dose. f) The messenger RNA (mRNA) levels of Gclc, Gclm, Ho-1, and Nqo1 were examined by quantitative polymerase chain reaction. g) The protein levels of GCS, HO-1, and NQO1 were tested by Western blot. h) Western blot results were quantified by ImageJ software (National Institutes of Health, USA). Data were presented as mean and standard deviation of three independent experiments. *p < 0.05 versus control of the same glucose concentration group, #p < 0.05 versus 5 mM glucose with the same DMF and/or RANKL treatment. COMPARE p < 0.05 versus the same glucose concentration plus RANKL treatment (two-way analysis of variance).
(5 mM or 40 mM) RAW 264.7 cells were treated with RANKL (50 ng/ml), DMF (20 μM), or co-treated with RANKL and DMF for 60 minutes. After being washed with PBS three times, cells were incubated with DCF at 37°C for 60 minutes and re-suspended in flow cytometry (FACS) buffer. The fluorescence was detected using a Flow cytometry system (BD Biosciences, USA) with an excitation wavelength of 488 nm and emission wavelength of 525 nm. The results were analyzed using FlowJoO software (TreeStar, USA).

**Western blot analysis.** Total proteins were extracted from the cells with radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technology (CST), USA). Protein concentrations were measured using the BCA Protein Quantitation Kit (Abbkine, USA). The same amounts of proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% polyacrylamide gels) and transferred onto polyvinylidene difluoride (PVDF) membranes (MilliporeSigma, USA). The membranes were blocked with 5% milk and incubated overnight at 4°C with primary antibodies from Santa-Cruz Biotechnology (USA) against β-actin (sc-1616), GCS (sc-390811), HO-1 (sc-390991), and NQO1 (sc-32793) and primary antibodies from CST against p-NF-κB (3033), NF-κB (3032), p-IkBα (8219), IkBα (9242), p-ERK (4370), ERK (4695), p-JNK (9251), JNK (9252), p-P38 (9215), P38 (9212), TRAF6 (8028), and RANK (4845). After that, the membranes were further incubated with horseradish peroxidase (HRP)-linked anti-rabbit immunoglobulin G (IgG) secondary antibody (1:3,000) in tris-buffered saline (TBST) for one hour. After being washed with TBST three times, the membranes were developed with enhanced chemiluminescence using enhanced chemiluminescence (ECL) reagents. The bands were visualized by the LI-COR Odyssey FC infrared imaging system (LI-COR, USA).

**Statistical analysis.** Data were presented as mean and standard deviation (SD), and analyzed using GraphPad Prism 7 (USA). The independent-samples t-test was used for comparison between two groups, and one-way or two-way analysis of variance (ANOVA) was used for comparison among multiple groups with Bonferroni post hoc tests. Differences with p < 0.05 were considered statistically significant.

**Results**

**DMF attenuates high glucose-induced susceptibility to osteoporosis in vivo.** To assess the effect of DMF on high glucose-induced osteoporosis, a glucose-related osteoporosis mouse model was established and treated with DMF. As expected, the blood glucose levels were over 16.7 mmol/l in the mice treated with streptozotocin, indicating that the diabetic mouse model was successfully established (p = 0.038, two-way ANOVA) (Supplementary Figure aa). The dual-energy X-ray absorptiometry and micro-CT analyses showed that streptozotocin-stimulated diabetic mice were more sensitive to OVX-induced osteoporosis, and DMF treatment significantly inhibited osteoporosis in the system (Supplementary Figures ab and ac) and reversed the levels of Tb.N, Tb.Th, Tb.Sp, and BV/TV (p = 0.015, two-way ANOVA) (Supplementary Figure ad). H&E and TRAP staining analyses showed that DMF effectively inhibited streptozotocin-enhanced osteoclast differentiation (Supplementary Figure ae to ag). In addition, the expression levels of bone metabolism biomarkers TRAP5b and CTX1 were enhanced in OVX-treated mice, and DMF inhibited these enhancements (p = 0.026, two-way ANOVA) (Supplementary Figure ah). These data suggest that DMF relieves high glucose-induced susceptibility to osteoporosis in vivo.

**DMF suppresses high glucose-induced osteoclast differentiation in vitro.** The role of DMF in high glucose-related osteoporosis was then explored in vitro. RAW 264.7 cells were treated with different concentrations of DMF. CCK-8 assays revealed that DMF treatment reduced RAW 264.7 cell viability in a dose-dependent manner with 80% survival at 75 μM (p = 0.016, two-way ANOVA) (Figure 1a), which was therefore selected as the dosage for subsequent experiments. As expected, glucose significantly increased TRAP accumulation in a dose-dependent manner, as demonstrated by the representative TRAP staining images (Figure 1b) and the increased count of TRAP-positive multinucleated cells containing > three nuclei from 50 in the control group to 150 in the DMF-treated group (p = 0.024, two-way ANOVA) (Figure 1c). Furthermore, glucose treatment upregulated the expression of Cathepsin K, Atp6v0d2, and H+-ATPase in a dose-dependent manner (p = 0.027, two-way ANOVA) (Figure 1d), indicating that glucose exposure induces osteoclast differentiation. Moreover, DMF suppressed the levels of TRAP promoted by glucose treatment in the RAW 264.7 cells as revealed by the representative images of TRAP staining (Figure 1e) and the count of TRAP-positive multinucleated cells containing > three nuclei (p = 0.011, two-way ANOVA) (Figure 1f). Meanwhile, the increased expression levels of Cathepsin K, Atp6v0d2, and H+-ATPase by glucose were reversed by DMF treatment (p = 0.015, two-way ANOVA) (Figure 1g). Actin filament (F-ACTIN) rings and bone pit analysis showed that DMF treatment also reversed the enhancing effect induced by high glucose levels (Figure 1k), suggesting that DMF inhibited osteoclast differentiation induced by high glucose in vitro.

**DMF inhibits high glucose-induced reactive oxygen species accumulation.** As high glucose can increase reactive oxygen species (ROS) accumulation, the impact of DMF on ROS levels in BMMs and RAW 264.7 cells treated with RANKL was further investigated. The results showed that DMF inhibited glucose-increased ROS levels under RANKL treatment in these cells (p < 0.05) (Figure 2e). Moreover, the expression levels of antioxidant enzymes Gclc, Gclm, Ho-1, and Nqo1 were elevated around five-fold by DMF treatment (p = 0.027, two-way ANOVA) (Figure 2f). Furthermore, DMF increased the expression levels of GCS, HO-1, and NQO1 in a dose-dependent manner (p = 0.031, two-way ANOVA) (Figure 2h). Together these data
N,N-Dimethylformamide (DMF) inhibits high glucose-caused osteoclast differentiation by targeting mitogen-activated protein kinase (MAPK) signalling and nuclear factor kappa B (NF-κB) signalling in bone marrow macrophages (BMMs). The glucose-treated BMMs were treated with receptor activator nuclear factor κB ligand (RANKL), DMF, or their combination for 30 minutes. a) Reactive oxygen species (ROS) levels were assessed by flow cytometry analysis (×200). b) Tartrate-resistant acid phosphatase (TRAP) staining was performed using an acid phosphatase kit, and the representative images of TRAP staining were shown. c) The count of TRAP-positive multinucleated cells containing > three nuclei was presented. d) The expression levels of cathepsin K, Atp6v0d2, and H+-ATPase were analyzed by quantitative polymerase chain reaction (qPCR) assays. e) and f) Following seven days of treatment with RANKL (50 ng/ml) and macrophage colony-stimulating factor (M-CSF) (30 ng/ml), cells were removed from the dentine discs, and resorption pits were visualized. g) The levels of RANK, TRAF6, p38, extracellular signal-related kinase (ERK), JNK, NF-κB (p65), IκBα, β-ACTIN and phosphorylated P38, ERK, JNK, NF-κB (p65), and IκBα were measured by Western blot. h) The results of Western blot analysis were quantified by ImageJ software (National Institutes of Health, USA). Data were presented as mean and standard deviation of three independent experiments. *p < 0.05 versus control of the same glucose concentration, #p < 0.05 versus 5 mM glucose with the same DMF and/or RANKL treatment, $p < 0.05 versus the same glucose concentration plus RANKL treatment (two-way analysis of variance). mRNA, messenger RNA.
suggest that DMF inhibited high glucose-induced ROS accumulation.

**DMF inhibits high glucose-activated MAPK and NF-κB signalling.** Next, the mechanism of DMF-induced protective effect against high glucose-related osteoporosis was investigated. Since MAPK pathway and NF-κB pathway play crucial roles in osteoclastogenesis and osteoclast differentiation, we speculated that DMF exerted its function by targeting MAPK and NF-κB signalling pathways. Western blot analysis revealed that DMF reduced high glucose-induced phosphorylation of P38, ERK, and JNK (p = 0.036, two-way ANOVA) as well as high glucose-induced phosphorylation of NF-κB (p65) and IkBα (p = 0.193, two-way ANOVA) (Supplementary Figure ba and bb). These results indicated that DMF inhibited high glucose-activated MAPK signalling and NF-κB signalling pathways.

**DMF inhibits high glucose-caused osteoclast differentiation by targeting MAPK signalling and NF-κB signalling in BMMs.** To further validate the inhibitory effect of DMF on high glucose-caused osteoclast differentiation, bone marrow macrophages (BMMs) were treated with DMF. The results showed that DMF reduced high glucose-promoted ROS levels in BMMs with RANKL treatment (p = 0.023, one-way ANOVA) (Figure 3a). As expected, DMF inhibited high dose glucose-enhanced accumulation of TRAP in BMMs, as demonstrated by the representative images of TRAP staining (Figure 3b) and the count of TRAP-positive multinucleated cells containing > three nuclei (p = 0.034, two-way ANOVA) (Figure 3c). Moreover, DMF treatment reversed high-glucose enhanced expression levels of Cathepsin K, Atp6v0d2, and H+-ATPase (p = 0.018, one-way ANOVA) (Figure 3d), suggesting that DMF inhibited high glucose-induced osteoclast differentiation. Furthermore, DMF treatment reversed the enhancing effect of high glucose levels on bone pits (Figure 3f) and attenuated high-glucose-activated phosphorylation of P38, ERK, JNK, NF-κB (p65), and IkBα (p = 0.029, two-way ANOVA) (Figure 3h), indicating that DMF relieved high glucose-caused osteoclast differentiation via targeting MAPK and NF-κB signalling pathways in BMMs.

**Discussion**

DM-OS is a severe disorder that affects increasing numbers of patients and is associated with multiple complications. As a multipurpose reagent, DMF has displayed potential biomedical activities in multiple diseases. For example, DMF has dual impacts on apoptosis and proliferation of breast cancer cells, depending on its concentration. It induces a characteristic change of cells and suppresses tumorigenesis in human colon carcinoma cells. DMF also inhibits cisplatin-related kidney injury by stimulating the Nrf2 pathway and repressing the NF-κB pathway. DMF affects Kdo glycosylation by stereoselective synthesis for α-Kdo glycosides, and regulates standard conversion potentials for oxygen and carbon dioxide couples. In this study, we found that DMF inhibits high glucose-induced susceptibility to osteoporosis in vivo and suppresses high glucose-induced osteoclast differentiation in vitro. These data demonstrated an unreported function of DMF in DM-related osteoporosis, providing new insights into the biomedical activity of DMF.

Previous studies have indicated that overdose of DMF causes hepatic cytotoxicity, which leads to the consideration of choosing the appropriate dose for disease treatment. Moreover, DMF is known to elicit certain alcohol intolerance reactions. Hence, appropriate application strategies should be designed, and patients with histories of alcohol abuse should be particularly counselled. Notably, our data showed no obvious toxicity of DMF to mice, possibly due to the low dose we adopted in this work.

ROS are required for cell signal transduction and other physiological functions, and are involved in modulating osteoporosis, including DM-related osteoporosis. Activation of ROS/MAPKs/NF-κB/NLRP3 and efferocytosis inhibition have been observed in osteoclast-related diabetes osteoporosis. Lourein B represses RANKL-stimulated ovariectomized osteoporosis and osteoclastogenesis by inhibiting the actions of NFATc1 and ROS. Pseurotin A represses osteogenesis and inhibits ovariectomized-produced bone loss via ROS. Vitamin B5 restrains RANKL-related osteogenesis and ovariectomy-induced osteoporosis by modulating ROS production. Sesamin preserves osteonecrosis-related femoral head by repressing ROS-induced osteoblast apoptosis in rat models. Angelica polysaccharide promotes proliferation and osteoblast differentiation of mesenchymal stem cells by regulating long non-coding RNA H19. Low concentration of dexamethasone influences osteoblast viability by provoking autophagy via mediating ROS. Irbesartan inhibits unconventional glycation end product-regulated damage in DM-OS by mediating ROS. In our work, we found that DMF suppresses glucose-stimulated ROS production and simultaneously elevates the levels of antioxidant enzymes including Gclc, Gclm, Ho-1, and Nqo1 in a dose-dependent manner. These data reveal that DMF inhibits high glucose-induced ROS accumulation and demonstrates a novel correlation of DMF with ROS in modulating DM-OS.

As the fundamental cellular signalling pathways, MAPK signalling and NF-κB signalling pathways are involved in the progression of osteoporosis, including DM-OS, and serve as potential targets for anti-osteoporosis treatment. It has been reported that quercurtin inhibits osteoporosis by controlling MAPK signalling in ovariectomized rats. Timosaponin All inhibits inflammation injury in Alloxan-stimulated diabetic osteoporosis in zebrafish AGEs-produced osteoblasts by regulating MAPK signalling. Taurine stimulates BMP-2/Wnt3a-related mineralization and osteoblast differentiation by regulating MAPK signalling. Bergapten exercises inhibitory influences on DM-OS by modulating NF-κB and JNK/MAPK signalling in vivo. Meanwhile, a previous study has identified that proanthocyanidins repress the formation and function of osteoclasts by restraining JNK and NF-κB signalling under...
osteoporosis treatment\textsuperscript{55}. Ferulic acid inhibits osteoporosis by stimulating SIRT1 and NF-κB in glucocorticoid-related osteoporosis neonatal rats\textsuperscript{56}. Icarin abolishes osteoclast formation by modulating RANKL-induced TRAF6/NF-κB/ERK signalling in Raw 264.7 cells\textsuperscript{57}. Static represses RANKL-stimulated osteoclastogenesis by inhibiting STAT3 and NF-κB activation\textsuperscript{58}. Inhibiting NF-κB signalling pathway by anthracycline glycoside aloin plays a part in the osteoclastogenesis\textsuperscript{59}. Tubemisolide I suppresses RANKL-stimulated NF-κB signalling, osteoclast formation, and diabetes-related bone loss in rats\textsuperscript{60}. Apolipoprotein E presents a critical function in preserving bone mass by increasing osteoblast differentiation via ERK1/2 pathway and by repressing osteoclast differentiation via NF-κB signalling\textsuperscript{61}. Coincidently, one previous study has demonstrated the function of DMF in oxidation and its correlation with NF-κB signalling\textsuperscript{62}. Therefore, we studied whether NF-κB signalling is involved in DMF-alleviated DM-OS, and obtained the expected results that DMF administration decreases phosphorylation of p38, ERK, and JNK signalling.

In conclusion, we found that DMF inhibits high glucose-induced osteoporosis by targeting MAPK and NF-κB signalling and uncovered a novel inhibitory effect of DMF on OS. The study indicates that DMF might serve as a potential candidate for DM-OS treatment.

Supplementary material

An ARRIVE checklist is included to show that the ARRIVE guidelines were adhered to in this study, as well as supplementary figures expanding on the study results.

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