Trehalose reduces bone loss in experimental biliary cirrhosis rats via ERK phosphorylation regulation by enhancing autophagosome formation

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Abstract

Bone loss is a severe complication of primary biliary cirrhosis (PBC). Trehalose was intermittently administered in bile duct-ligated (BDL) male rats, a PBC-related osteoporosis model, for 4 weeks to reduce osteoporosis. Femoral bones were assessed ex vivo by micro computed tomography (CT) and histomorphometry. The potential mechanisms related to the reduction of osteoporosis were explored by evaluating the effect of trehalose on osteoblast autophagy, osteogenesis, osteoclastogenesis, and ERK phosphorylation. The results demonstrated that trehalose reduced osteoporosis of BDL rats and decreased osteoblast-mediated osteoclast differentiation by enhancing osteoblast autophagy to regulate osteoprotegerin (OPG) secretion. Hydroxychloroquine (HCQ) increased the expression of OPG and OPG/receptor activator genes for nuclear factor-κB ligand (RANKL) ratio, and reduced osteoblast-mediated osteoclastogenesis by inhibiting autophagy flux and inducing autophagosome formation. Furthermore, trehalose increased the phosphorylation of ERK1/2 in MC3T3-E1 cells, and the ERK inhibitor PD98059 reversed the upregulation of OPG gene and reduction of trehalose-induced osteoclastogenesis. The treatment with HCQ markedly increased the ERK phosphorylation. The correlation between autophagosome formation and ERK phosphorylation was confirmed in autophagy proteins (ATG) 4B or ATG5-deficient cells. Thus, trehalose could decrease osteoblast-mediated osteoclastogenesis and reduce PBC-related bone loss by regulating ERK phosphorylation via autophagosome formation.

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Abbreviations: ATG, autophagy-associated gene; ATF4, activating transcription factor 4; BDL, bile duct-ligated; BMD, bone mineral density; BV/TV, bone volume fraction; ERK, extracellular-regulated protein kinases; HCQ, hydroxychloroquine; LC3, microtubule-associated protein 1 light chain 3; OB, osteoblast; OC, osteoclast; OPG, osteoprotegerin; OPN, osteopontin; PBC, primary biliary cirrhosis; RANKL, receptor activator for nuclear factor-κB ligand; Runx2, runt-related transcription factor 2; SO, sham operation; siRNA, small interfering RNA; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; Tb.N, trabecular number.

Xingquan Xu and Rongliang Wang are contributed equally to this work.

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INTRODUCTION

Osteoporosis is one of the most severe complications of primary biliary cirrhosis (PBC), which results in an increased risk of fracture. The incidence of osteoporosis in patients with cirrhosis is between 12% and 55%, and the incidence of fracture varies from 5%-20%. A meta-analysis, designed to comprehensively analyze the association between PBC and osteoporosis, demonstrated that PBC patients might suffer from osteoporosis. Previous studies demonstrated that the risk of bone fracture among patients with PBC was higher as compared to that in the general population. To date, the etiology of bone loss in PBC patients is elusive. Some factors that affect bone metabolism may result in the pathogenesis of osteoporosis in PBC patients, including vitamin K deficiency, vitamin D and calcium metabolism alterations, deficiency of insulin-like growth factor 1 (IGF-1), and the release of cytokines. A large number of studies have shown that low bone turnover rate may lead to lesser mean wall thickness, a defect in matrix synthesis, and low bone formation rate, primarily contributing to the mechanisms of osteoporosis in PBC patients. These studies showed that both reduced bone formation and increased bone resorption might be associated with PBC-related bone loss.

Currently, there is no specific therapy for PBC-related bone loss. For the prevention of bone loss in PBC, the use of bisphosphonates, vitamin D, and calcium supplementation or estrogen is recommended. However, these agents failed to show definite beneficial effects. Bisphosphonate is the first-line treatment in postmenopausal osteoporosis but is shown to have little effect on PBC, although some studies demonstrated conflicting results. Unequivocal data confirming the efficacy of calcium and vitamin D supplements for preventing bone loss in PBC patients are yet lacking. Moreover, hormone replacement therapy exerts controversial effects and can worsen cholestasis. Therefore, identifying the effective agents that prevent bone loss in PBC is imperative.

The agents that are related to increased bone formation or reduced bone resorption may be effective in treating PBC-related bone loss. The mitogen-activated protein kinase (MAPK)-extracellular signal regulated kinase (ERK) signaling pathway is involved in osteoblastogenesis and osteoclastogenesis, and plays a critical role in the activation of various growth factors. In a previous study, we showed that trehalose, a natural disaccharide, plays a critical role in the activation of various growth factors. Trehalose is known to be an autophagic activator and re-enhances the healing of pathological delay in the bone fracture union. Trehalose has favorable effects on the bone mass in PBC.

In the present study, a bile duct-ligated (BDL) male rat model was used to establish the features of biliary cirrhosis and the characteristics of osteoporosis, as described previously. The effect of intraperitoneal injection of trehalose on bone mass in BDL male rats and the related mechanisms were investigated.

MATERIALS AND METHODS

2.1 Materials and reagents

D-(-)-trehalose dehydrate (Figure 1A, purity ≥ 99%) and LC-3 antibody were obtained from Sigma-Aldrich (St Louis, MO, USA). Hydroxychloroquine (HCQ) was procured from Aladdin (Shanghai, China). PD98059 was from MedChem Express (Monmouth Junction, NJ, USA). Recombinant mouse macrophage colony-stimulating factor (M-CSF) and recombinant mouse RANKL were purchased from R&D Systems (Minneapolis, MN, USA). The primary antibodies against Runx2, OPN, and OPG were purchased from Abcam (Cambridge, UK). The goat anti-rabbit HRP-labeled secondary antibody was obtained from Fude Biological Technology (Hangzhou, China). Beclin1 and ATF4 antibodies were from Proteintech Group (Chicago, IL, USA). The antibodies against p-ERK, ERK, and GAPDH were from Cell Signaling Technology (Danvers, MA, USA).

2.2 Animals

A total of 30 male Sprague Dawley (SD), 8-week-old rats, were purchased from the Laboratory Animal Center of Drum Tower Hospital, Nanjing, China. Before the experiments, all animals were housed in regular cages at 24°C under a 12-hours light/dark cycle for 1 week. The rats were allowed free access to food and water during the whole study.

2.3 Experimental protocol

All animal procedures were approved and carried out in accordance with the guidelines of the Ethics Committee of Drum Tower Hospital, Medical School of Nanjing University, Nanjing, China (Ethics approval number: 20160902).

The experimental rats were equally and randomly divided into three groups: sham operation (SO), BDL, and BDL + trehalose (Tre) groups. The rats in the last two groups underwent BDL as described previously (Figure 1B). After the operation, the BDL-rats were left untreated for 2 weeks and then, treated
from the third postoperative week intermittently with either trehalose (2 g/kg) three times/week or a vehicle for 4 weeks. Blood samples were withdrawn from the animals, 1 day prior to death, for biochemical analysis. The rats were killed by intraperitoneal injection of 10% chloral hydrate. The livers and lower limbs were harvested for histological assessment.

2.4 | Microcomputed tomographic (micro-CT) analysis

The distal femurs of the rats were scanned using a micro-CT system (µCT-80, Scanco Medical, Bassersdorf, Switzerland), and morphometric 3D analysis was conducted after imaging. The X ray-tube was set at 70 kV and 114 mA at a resolution of 17.5 μm with an integration time of 250 ms. Finally, the region of interest (ROI) encompassing 5% of femoral length 0.05 mm below the growth plate was selected to determine several bone measurements: bone mineral density (BMD), bone volume fraction (BV/TV), trabecular thickness (Tb.Th), and trabecular number (Tb.N).

2.5 | Histopathological analysis

The knee joints were fixed in 4% (v/v) paraformaldehyde for 24 hours. Then, the specimens were decalcified in 5% (v/v) EDTA for 1 month. Following dehydration and clearing, the tissues were embedded in paraffin and sliced into 3-μm sagittal sections. The slides of each joint were stained with safranin O, Masson, and TRAP staining.

2.6 | Immunohistochemistry (IHC) analysis

Bone sections were rehydrated, and endogenous peroxidase was blocked by 3% hydrogen peroxide. The sections were incubated with 0.4% pepsin (Aladdin, Shanghai, China) in 0.1 mM HCl at 37°C for 60 minutes for antigen retrieval. Subsequently, the sections were blocked with 5% bovine serum albumin for 60 minutes, and incubated with primary antibody overnight at 4°C, followed by anti-rabbit secondary antibody for 3 hours. Finally, the sections were developed with chromogen for 10 minutes at 37°C, dehydrated, and mounted for examination.
2.7 | Cell cultures

Mouse calvarial pre-osteoblast cell line MC3T3-E1 was purchased from the Institute of Life Science Cell Culture Center (Shanghai, China) and maintained in alpha-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) and penicillin (100 U/mL) at 37°C in a 5% CO₂ atmosphere.

2.8 | In vitro osteoclast (OC) formation

Bone marrow nucleated cells were obtained from femurs of 8-week-old mice and incubated in α-MEM, 10% FBS, and 30 ng/mL M-CSF for 1 day. The non-adherent cells (2 × 10⁵ cells/well) were plated in 24-well plates with the osteoclastogenic medium (50 ng/mL RANKL and 30 ng/mL M-CSF) for 8 days. OCs were detected using TRAP kit (Sigma-Aldrich, MO, USA) and TRAP-positive multinucleated cells were viewed as mature OC.

2.9 | Transwell co-culture

The co-culture system (Figure 2D) was established using Transwell culture kits (Corning 24-well plates, 0.4 μm, Thomas Scientific, NY, USA). Briefly, MC3T3-E1 cells were cultured at a density of 5 × 10⁵ cells/well in the upper wells under specific treatment, and 2 × 10⁵ cells/well bone marrow macrophages were seeded in the lower wells containing the osteoclastogenic medium. After co-culturing for 5 days, TRAP staining was performed.
2.10 Western blotting

The treated cells (5 × 10^4 cells/cm²) were rinsed with phosphate-buffered saline (PBS) and lysed in radioimmunoprecipitation assay (RIPA) buffer. An equivalent of 20 μg of samples was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 4% nonfat milk-TBST solution for 1 hour and probed with the appropriate primary antibodies overnight at 4°C, followed by incubation with secondary antibody. Finally, the immunoreactive bands were detected with an Enhanced Chemiluminescence Kit (Fude Biological Technology, Hangzhou, China) using the Tanon-5200 system (Biotanon, Shanghai, China).

2.11 Quantitative real-time PCR

The MC3T3-E1 cells were plated in 6-well plates at a density of 3.5 × 10^5/mL. After incubation for 24 hours, total RNA was extracted using the RNA-Quick Purification Kit (ES Science, Shanghai, China) and cDNA was generated with a HiScriptIIQ RT SuperMix for qPCR (Vazyme Biotech, Nanjing, China), followed by analysis using a ChamQTM SYBR Color qPCR Master Mix (Vazyme Biotech). The amplification and detection were carried out on a LightCycler 480-II (Roche, Mannheim, Germany). The primers are listed in Table 1. The levels of the target transcript were normalized to that of the internal reference (2−ΔΔCT method).

2.12 Autophagy determination

The 3 × 10^4 MC3T3-E1 cells/well were seeded in 24-well plates and transiently transfected with mRFP-GFP-LC3 adenoviral vectors (HanBio, Shanghai, China) using Lipofectamine 3000 (Invitrogen) according to the manufacturer’s protocol. Then, the cells were incubated in growth medium with the adenoviruses for 6 hours at 37°C, followed by that in medium containing trehalose or vehicle for an additional 24 hours at 37°C. Finally, the cells were analyzed by a fluorescence microscope (Zeiss Microsystems, Gottingen, Germany). The autophagic flux was quantified by enumerating the number of GFP and mRFP puncta/cell.

2.13 Transmission electron microscopy

The 5 × 10^5 MC3T3-E1 cells/well were harvested by centrifugation at 1000 × g for 5 minutes at 4°C and fixed overnight with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). All the specimens were washed in 1% aqueous tetroxide in 0.1 M sodium cacodylate (pH 7.4) for 2 hours and stained with 2% aqueous uranyl acetate for 2 hours. After dehydration in an ethanol series, the samples were embedded into a sheet and sliced into ultra-thin sections (90-nm-thick) with a Leica UC6 using a diamond knife. Subsequently, the sections were counterstained with 2% saturated uranyl acetate and examined under an H-7650 electron microscope (Hitachi, Japan).

2.14 siRNA transfection

The following siRNAs were designed and synthesized (Hippobio, Huzhou, China): siATG4B-1 (sense, s) 5′-GCAUCGAUUCUACAGAUUTT-3′, (antisense, as) 5′-AAUCAUGUAGAAUCGAGCAT-3′; siATG4B-2 (s) 5′-CCACAUACUCAUGUAATT-3′, (as) 5′-UUACUA CAUGAGUAUGUGGT-3′; siATG5-1 (s) 5′-GCGGUUG AGGCUACUUATT-3′, (as) 5′-UUAAUGUGACCUCUA CCAGCAT-3′; siATG5-2 (s) 5′-CCGGAAACUCAUGGA AUUATT-3′, (as) 5′-AAUAUCCUGAUUGUUCGTTT-3′. The MC3T3-E1 cells were cultured in 6-well plates at a density of 2 × 10^5/mL for 24 hours to 60%-70% confluency and transfected using Lipofectamine 3000 siRNA reagent. The silencing efficiency was determined by qPCR analysis on the mRNA expression of the target genes.

### Table 1

| Prime      | Forward (5′-3′)       | Reverse (5′-3′)       |
|------------|-----------------------|-----------------------|
| ATF4       | CTCCTGACCAGCCTGATGAC  | CAACTCCACTGCCTAGCTTAA |
| OPN        | TACGACATGAGATGGCAGTA  | TATAGGATCTGGTGAGCATGAA |
| OPG        | CTTTGCCGCTGACCTCTAT   | CACACACTGCCTTGGTTG   |
| RANKL      | AAGTCACCTGTCCCTTGGTA  | GATAGTCTGGTAGGTAGCCT |
| β-actin    | CATGTACGTTGCTTCTTCCAGG | CTCCTTAATGTCAGCACAGCAGAT |

Abbreviations: ATF4, activating transcription factor 4; OPN, osteopontin; OPG, osteoprotegerin; RANKL, receptor activator for nuclear factor-κB ligand.
2.15 | Statistical analysis

All numerical data are represented as mean ± standard error of the mean (SEM). The statistical analysis of the parameters among the groups was performed using SPSS statistical software program 20.0 (IBM, Armonk, NY, USA), and one-way analysis of variance (ANOVA) was used to determine the statistical significance. *P*-value ≤ .05 was considered statistically significant.

3 | RESULTS

3.1 | Animals

Some rats died before the end of the study because of severe complications, such as infection, cirrhosis ascites, and emaciation (one in the SO group, three in the BDL group, and three in the BDL + TRE group). The present study enrolled nine rats in the SO group, seven rats in the BDL group, and seven rats in the BDL + Tre group.

3.2 | Effects of trehalose on the BDL model in vivo

The 3D reconstruction evaluation of the femoral trabecular bone structure was significantly different between the BDL and SO rats, and the administration of trehalose increased the bone structure in BDL rats (Figure 1C). According to the micro-CT imaging analysis, trehalose treatment restored the BMD to the level similar to that in the SO group. Although no significant change was detected in Tb.N, an increase in BV/TV and Tb.Th was observed after trehalose administration (Figure 1D). The levels of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and type IV collagen (COL IV) in the BDL mice were significantly increased as compared to those in the SO mice. However, trehalose treatment significantly reduced the serum levels of ALT, AST, and COL IV, while serum osteocalcin was significantly higher in BDL rats as compared to the levels in the SO and BDL groups (Supplemental Table 1). Histological analyses evaluated the therapeutic effects of trehalose in these animals (Figure 1E). Glicosaminoglycans (GAGs) contributed to osteoblast (OB) differentiation, and staining with saframin O/fast green showed that the level of GAGs adjacent to the cartilage was reduced in the BDL model group as compared to that in the SO group. In addition, the collagen in the BDL group exhibited sparsely arranged Masson staining. Simultaneously, the number of TRAP-positive (TRAP+) OCs in the BDL group was significantly higher than that in the SO group (Figure 1F). In the trehalose treatment group, GAGs showed intense and uniform staining with saframin O, and Masson staining showed an increase in the amount of trabecular bone and a decrease in the trabecular space. Nevertheless, the number of TRAP+ OCs on trabecular bone was decreased with the treatment of trehalose. Compared to the SO rats, BDL rats showed obvious changes in the liver morphology, including disordered arrangement/necrosis of liver cells, marked fibrosis in portal tracts, diffused high bile duct hyperplasia, and severe portal infiltrates. These changes in the liver morphology were markedly improved by trehalose treatment (Supplemental Figure 1).

3.3 | Effects of trehalose on osteoblast (OB)-mediated OC differentiation in vivo and in vitro

For the in vitro study, trehalose treatment modestly increased the level of runt-related transcription factor-2 (Runx2) in MC3T3-E1 cells (Figure 2A, Supplemental Figure 3A). Also, a significant increase in the expression of ATF4, OPN, OPG, and OPG/RANKL mRNA in response to trehalose exposure was detected in a concentration-dependent manner (Figure 2B). In order to elucidate the mechanism of the osteogenesis effects of trehalose in vivo, the expression of ATF4, OPN, and OPG was detected by IHC. The trabecular bone of the BDL group showed a decrease in the positive staining for ATF4, OPN, and OPG as compared to the SO group, while trehalose administration markedly reversed the staining of these proteins (Figure 2C). The prolonged trehalose exposure did not increase the ALP activity and mineral deposition than those observed in untreated controls (Supplemental Figure 2). However, trehalose resulted in a significant decrease in OC differentiation as evident from TRAP staining in transwell culture systems (Figure 2E,F).

3.4 | Effects of trehalose on autophagy in vivo and in vitro

The IHC analysis of LC3 showed that OBs, the cells attached to the surface of the bone trabeculae, exhibited less positive staining in the BDL group than that in the SO group, while the administration of trehalose increased the amount of positive staining on the surface of the bone trabeculae as compared to that in the BDL group (Figure 2C). Furthermore, Western blot analysis showed a significant elevation in the expression of autophagy-associated proteins, Beclin1, and LC3II/I, in trehalose-treated OBs in a dose-dependent manner (Figure 3A). Interestingly, trehalose-induced activation of autophagy peaked at 24 hours (Supplemental Figure 3B,C, and Figure 3B). Furthermore, the number of typical autophagosomes with double membranes was increased in the trehalose-treated group as compared to that in the control group,
as observed by transmission electron microscopy (Figure 3C). After infection with the GFP-RFP-LC3 adenovirus, treatment with trehalose enhanced both green and red staining, and colocalization elevated by the yellow puncta, thereby confirming the activation of autolysosomes and autophagy flux (Figure 3D,E).

### 3.5 Effects of autophagy on OB-mediated OC differentiation in vitro

The HCQ treatment attenuated the changes in Beclin1 and increased the LC3II/I level induced by trehalose (Figure 4A). Electron microscopy revealed less autophagolysosome formation in the cytoplasm, but large autophagosomes as a result of HCQ treatment (Figure 4F). The infection with the tandem GFP-RFP-LC3 adenovirus confirmed that the lysosomal acidification inhibitor HCQ increased the yellow and green puncta in MC3T3-E1 cells, while the red punctate staining decreased, indicating that the production of autophagosomes was upregulated and the trehalose-induced autophagy flux was inhibited by HCQ (Figure 4D,E). However, enhanced osteogenesis occurred in the HCQ-treated group, as the expression of Runx2 and other relative mRNAs was increased as compared to those observed in the trehalose-treated group (Figure 4B,C). In addition, HCQ treatment also significantly repressed the OC formation of bone marrow macrophages (Figure 4G,H).

### 3.6 Effects of trehalose on ERK signaling and OB-mediated OC differentiation in vitro

Short-term exposure to trehalose robustly enhanced the ERK1/2 phosphorylation in MC3T3-E1 cells at 15 minutes.
Although trehalose treatment increased ERK phosphorylation at all time points, prolonged trehalose exposure did not increase the effect to levels greater than those observed at 15 minutes in a time-dependent decreasing trend (Figure 5A). However, this enhancement was reversed by the ERK inhibitor PD98059 (Figure 5B). Furthermore, PD98059 also reversed the expression of Runx2 protein and that of ATF4, OPN, OPG, and OPG/RANKL ratio mRNAs in trehalose-treated cells (Figure 5C,D). Analysis of coculture models confirmed an increase in OC differentiation...
because of PD98059 treatment in trehalose-treated group (Figure 5E,F).

3.7 Trehalose-induced autophagosomes regulate ERK phosphorylation and OB-mediated reaction in vitro

The bidirectional crosstalk between ERK and autophagy effectuated the HCQ-mediated increase in ERK phosphorylation as compared to that in trehalose-treated cells (Figure 6A). The involvement of autophagosomes induced by trehalose in ERK phosphorylation and osteogenesis was assessed based on the partial loss of ATG4B, which played a vital role in the recycling of LC3II to LC3I and increased the LC3II levels irrespective of trehalose treatment (Figure 6C). In addition, augmenting LC3II in ATG4B-deficient cells enhanced p-ERK and Runx2 expression levels in the trehalose-treated group (Figure 6B). ATF4, OPN, OPG, and OPG/RANKL ratio were remarkably decreased in ATG5-silenced cells under trehalose exposure (Figure 6E, G). In addition, ATG4B/ATG5-siRNAs successfully confirmed the above experimental results and excluded the off-target effects (Supplemental Figures 4-7).

4 DISCUSSION

The serological tests, histological study, and micro-CT results revealed the clinical features of biliary cirrhosis in the animal model. Similarly, some studies demonstrated reduced bone formation and increased bone resorption in BDL male rats, which is in accordance with the PBC patients. The BDL male rat model used in the present study was simple and effective, as described previously. Many scholars considered that cholestasis is one of the major risk factors of impaired bone formation. However, a study showed that...
FIGURE 6 Trehalose-induced autophagosomes regulate ERK phosphorylation and OB-mediated reaction in vitro. A, The autophagy inhibitor increased the effects of trehalose on ERK phosphorylation in MC3T3-E1 cells (n = 3). B, ATG4B depletion (siATG4B-1) increased the trehalose-induced ERK phosphorylation after the transfection of MC3T3-E1 cells with scrambled siRNAs (Scr) or siRNAs against ATG4B in the presence/absence of trehalose (n = 3). C, ATG4B deficiency (siATG4B-1) augmented trehalose-induced Runx2 expression and autophagic LC3-II levels (n = 3). D, siATG4B-1 cells increased the expression of ATF4, OPN, OPG, and OPG/RANKL ratio after exposure to trehalose (n = 3). E, The expression of Runx2 and LC3-II in MC3T3-E1 cells was inhibited by siATG5-1 under trehalose exposure (n = 3). F, ERK phosphorylation in siATG5-1 cells was markedly impaired as compared to cells transduced with Scr after treatment with trehalose (n = 3). G, siATG5-1 blocked the advance of ATF4, OPN, OPG, and OPG/RANKL ratio induced by trehalose (n = 3).
using ursodeoxycholic acid to improve cholestasis did not lead to an increase in bone mass. The lack of correlation between improvement of cholestasis and bone mass suggested that agents that ameliorate cholestasis might not be suitable to treat the bone loss in PBC. Another study demonstrated that intermittent subcutaneous administration of human parathyroid hormone 1-34 (hPTH 1-34) to BDL male rats effectively prevented the bone loss. Herein, we hypothesized that agents that promote bone formation or decrease bone resorption could be considered in managing PBC-related osteoporosis.

The present study demonstrated that trehalose prevented bone loss both in vivo and in vitro. Decreased trabecular bone volume and thickness were observed in the distal femurs of BDL male rats, which could be rescued by trehalose administration. The expression of Runx2 protein and that of ATF4, OCN, and OPN genes was upregulated after treatment of MC3T3-E1 cells with 50 mM trehalose, which illustrated that trehalose may increase osteoblastogenesis at an early stage. However, we did not observe positive alkaline phosphatase staining after trehalose treatment. Alizarin red staining even indicated that trehalose inhibited calcium deposition. The trehalose effect of preventing bone loss may not be related to enhanced osteoblastogenesis. Moreover, trehalose treatment suppressed the enhancement of TRAP + OCs in BDL-rat bones. The expression of OPG and OPG/RANKL ratio was upregulated in MC3T3-E1 cells after treatment with 50 mM trehalose. The Transwell culture systems showed that trehalose-treated MC3T3-E1 cells significantly decreased the OC differentiation as evident by TRAP staining. The data indicated that trehalose suppressed osteoclastogenesis by mediating the OPG expressed by OBs. OPG was accepted to regulate the OC formation and protect the skeleton from excessive bone resorption by binding to RANKL and preventing it from binding to its receptor, RANK. Thus, the present study showed that trehalose reduced the bone loss of PBC rats via decreasing OC differentiation and reducing bone resorption.

Trehalose, a natural non-reducing disaccharide present in diverse organisms, is widely used in the food and drug storage industry. To the best of our knowledge, only a few studies have assessed the effect of trehalose on bone mass. A study showed that the delivery of BMP-2 lyophilized with trehalose reduces the use of BMP-2 in bone regeneration. However, whether trehalose served only as a stabilizer or as an agent to induce bone regeneration was not yet been determined. Some studies have reported that trehalose prevents OC differentiation in a rat periodontitis model. Also, it suppresses the lipopolysaccharide-induced osteoclastogenesis bone marrow in mice. The results of these studies were similar to those of the present study.

Furthermore, trehalose decreased osteoclastogenesis by inducing autophagy in OBs to secret OPG. In recent years, trehalose has been widely accepted as an autophagy inducer. It has been shown to play a pivotal role in managing various types of diseases via autophagy. These studies showed that trehalose enhances autophagy in several cell types, including motoneurons, fibroblasts, aortic artery endothelial cells, and keratinocyte cell lines. However, no study has yet determined whether trehalose could induce autophagy in OBs. Impaired autophagy in the femurs of BDL male rats was observed, which was alleviated by trehalose administration. Simultaneously, the present study, for the first time, showed that autophagy is probably involved in the pathogenesis of osteoporosis in PBC. We also demonstrated that treatment with 25 or 50 mM trehalose increased the ratio of LC3II/I

**FIGURE 7** Schematic illustration of the proposed mechanism. Hypothetical signaling pathways involved in trehalose-mediated regulation of autophagosome-induced ERK phosphorylation and OB-mediated OC differentiation in MC3T3-E1 cells.
in MC3T3-E1 osteoblast cells, indicating the activation of autophagy. RFP-GFP-LC3 double-fluorescence staining and transmission electron microscopy further confirmed the results. The correlation between autophagy and bone formation has been confirmed previously. Moreover, autophagy deficiency in OBs triggered an imbalance between OBs and OCs, resulting in a low bone mass. A review described the potential therapeutic role of autophagy in glucocorticoid-induced osteoporosis. Herein, trehalose-induced autophagy in MC3T3-E1 cells increased the expression of Runx2 protein and upregulated the expression of ATF4, OPN, OPG, and OPG/RANKL ratio. 50 mM trehalose-treated MC3T3-E1 showed significantly suppressed OC differentiation. In addition, we found that HCQ inhibited the autophagy flux and increased the LC3II/I level. Enhanced OPG expression and decreased osteoclastogenesis were observed after HCQ treatment, indicating the correlation between a high LC3II/I level and decreased bone resorption.

We further observed that decreased osteoclastogenesis was mediated by ERK phosphorylation via increasing the autophagosome formation. The present study demonstrated that short-term exposure to trehalose enhanced the phosphorylation of ERK1/2 in MC3T3-E1 cells, which was reversed by the ERK inhibitor PD98059. Additionally, PD98059 rescued osteoclastogenic suppression effect of trehalose, indicating that ERK1/2 phosphorylation reduced bone resorption. Several studies demonstrated that ERK regulates OC differentiation, which was consistent with the results of the present study. Moreover, HCQ markedly increased the p-ERK levels, indicating an association between autophagosomes and ERK1/2 phosphorylation.

Thus, we verified the function of autophagic structures determining the degree of ERK phosphorylation and osteoclastogenesis. Accumulating evidence showed that silencing ATG4B in MC3T3-E1 cells increased the LC3II level, leading to an upregulation of ERK1/2 phosphorylation and significantly promoting the expression of OPG and OPG/RANKL ratio. However, contradictory results were found by silencing ATG5 in MC3T3-E1 cells. Therefore, it could be deduced that LC3-II-positive autophagosomes serve as scaffolds or cellular signaling platforms that facilitate ERK phosphorylation to decrease OC differentiation. ATG5 knockout significantly decreased the expression OPG/RANKL ratio; however, we observed the unstable results of ERK phosphorylation and OPG expression in ATG5−/− cells, which suggested that ATG5−/− LC3-II-deficient cells utilize gamma-aminobutyric acid type A receptor-associated protein (GABARAP)-positive structures as cellular platforms for ERK phosphorylation.

ERK mediated various physiological functions, including proliferation and autophagy. However, the current study showed that ATG regulates ERK phosphorylation. A previous study showed that ATG regulate the ERK phosphorylation. Another study showed that the activation of autophagy increases the p-ERK1/2 level and salvages the disinhibition of hippocampal neurons. In addition, we speculated that aberrant autophagy potentially links the decreasing ERK phosphorylation to the development of osteoporosis in biliary cirrhosis, and manipulating the formation of autophagosomes could be a therapeutic strategy for the treatment of a variety of diseases caused by altered ERK activity.

Nevertheless, the present study has several limitations. First, the number of animals enrolled in this study was small. Second, we did not conduct a study to identify the effect of trehalose on liver damage as our results showed that trehalose had little therapeutic effect on liver fibrosis. Third, the effect of oral administration of trehalose was not investigated as it is digested into glucose molecules in the mammalian digestive tract.

In conclusion, impaired autophagy might contribute to PBC-related bone loss. Trehalose showed a remarkable positive effect on the bone mass when administered to BDL male rats, a rodent model of PBC-related bone loss. Trehalose decreases the OB-mediated osteoclastogenesis and reduces the PBC-related bone loss by regulating ERK phosphorylation via autophagosome formation.

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CONFLICT OF INTEREST
The authors have declared no conflicts of interest.

AUTHORS CONTRIBUTIONS
Conception and design: X. Xu, D. Shi, and Q. Jiang; Animal procedures: X. Xu, R. Wu, and W. Yan; Micro CT analysis: W. Yan and T. Shi; Cell experiments: R. Wang; Data analysis and manuscript writing: X. Xu and R. Wang; Final approval of manuscript: All authors.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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