



Original Article



Inhibition of Postmenopausal Osteoporosis in Ovariectomized Mice by Huo Xue Tong Luo Capsule Using Network Pharmacology-based Mechanism Prediction and Pharmacological Validation

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Abstract

Background and objectives: Huo Xue Tong Luo Capsule (HXTL) has been clinically used to treat osteonecrosis of the femoral head, osteoporosis, and other bone and joint diseases with promising effects. Our previous study has shown that HXTL can promote osteogenesis in mesenchymal stem cells by inhibiting lncRNA-Miat expression through histone modifications. However, the mechanism by which HXTL treats postmenopausal osteoporosis (PMOP) remains unclear. In this study, we used network pharmacology-based mechanism prediction, molecular docking, and pharmacological validation to investigate the mechanism of HXTL in treating PMOP.

Methods: The key candidate targets and relevant signaling pathways of HXTL for PMOP treatment were predicted using network pharmacology and molecular docking analysis. RAW264.7 cells were used for Western blot to validate the predicted mechanistic pathways. The ovaries of mice were surgically removed to simulate PMOP. The effect of HXTL on PMOP was evaluated using tartrate-resistant acid phosphatase staining and immunohistochemical assays *in vivo*.

Results: Network pharmacology analysis suggested that HXTL interacted with 215 key targets linked to PMOP, primarily affecting the PI3K-AKT signaling pathway. Molecular docking showed that the main components of HXTL exhibited strong binding affinity to NFATc1, p-PI3K, and p-AKT1. Furthermore, our *in vitro* results confirmed that HXTL suppressed the PI3K-AKT signaling pathway. *In vivo*, HE and tartrate-resistant acid phosphatase staining results showed that HXTL inhibited osteoclast formation and protected bone mass.

Keywords: Huo Xue Tong Luo Capsule; Post-menopausal osteoporosis; Network pharmacology; Molecular docking; PI3K-AKT signaling pathway; Osteoclastic differentiation.

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Conclusions: This research demonstrated that HXTL could inhibit osteoclast formation and prevent bone loss induced by ovariectomy in mice by inhibiting the PI3K-AKT signaling pathway. These findings provide important evidence for the clinical application of HXTL in treating PMOP.

Introduction

Osteoporosis (OP) is a chronic metabolic orthopedic disorder characterized by a reduction in bone mass, a loss of organic components of bone, and damage to bone structure, which is more common in the elderly. In general, the process of bone loss is

asymptomatic but increases the risk of fractures, impairs bone mobility, and reduces function, thereby lowering the patient's quality of life. Dual-energy X-ray bone densitometry, which is used to measure bone mineral density, is an important diagnostic tool for OP.¹ In addition, the risk of osteoporosis increases with age. Life expectancy in modern societies has risen, leading to a growing population of older individuals and, consequently, more cases of osteoporosis and fractures, especially among women.² Post-menopausal osteoporosis (PMOP) is caused by the significant decline in estrogen levels in women after menopause, which leads to osteoclast proliferation and differentiation, disrupted bone metabolism, and a reduction in bone mass. Currently, treatments for osteoporosis typically include parathyroid hormones, bisphosphonates, and calcitonin.³ Despite their effectiveness, these therapies are limited by high costs, poor patient compliance, and potential side effects. Osteoporosis is therefore a major public health challenge, necessitating the development of more effective, safer, and affordable prevention and treatment options than traditional medicines.⁴

In traditional Chinese medicine, osteoporosis is referred to as “bone blight” which is attributed to kidney deficiency, spleen deficiency, blood stasis, and other factors leading to bone degeneration and marrow reduction. Relevant experimental studies suggest that tonifying the kidneys should begin by promoting blood circulation and removing blood stasis.⁵ Traditional Chinese medicine (TCM) holds a unique advantage in preventing the outcomes of OP due to its approaches of distinguishing and treating syndromes and its holistic perspective. For thousands of years, TCM prescriptions have been widely used in clinical practice to treat this disease.⁶ The prevention and treatment of OP in TCM mainly revolve around the theory that “the kidney is the main source of bone and bone marrow” with drugs primarily used to tonify the kidney, promote blood circulation, strengthen the spleen, and warm the body. Several traditional Chinese medicine formulas have been used for the prevention and treatment of OP, such as Jintiang capsule,^{7,8} Xianling Gubao capsule,^{9,10} and Bushen Jiangu capsule.¹¹ Based on clinical symptoms, Chinese medicine and its preparations can be combined appropriately to maximize their advantages of multi-component, multi-target, multi-pathway, and multi-action. This approach reduces drug toxicity while improving therapeutic efficacy, which has attracted considerable attention from researchers.

The phosphoinositide 3-kinase (PI3K)/AKT signaling pathway represents a complex network of enzymes and molecules that regulates various cellular processes, including proliferation, differentiation, apoptosis, and inflammation.¹² In bone biology, the PI3K/AKT signaling pathway has been implicated in the regulation of osteoblasts (bone-forming cells) and osteoclasts (bone-resorbing cells). Emerging evidence suggests that disruptions in PI3K/AKT signaling contribute to the imbalance between bone formation and resorption, leading to the development and progression of osteoporosis.¹³ In osteoclasts, activation of PI3K leads to the phosphorylation of AKT, and active AKT further influences downstream factors like NF- κ B, which are essential for osteoclast functionality.¹⁴ Moreover, the PI3K/AKT pathway also regulates the mobility and maturation of osteoclast precursors, which are crucial for the positioning and activity control of osteoclasts during bone remodeling processes.¹⁵

Huo Xue Tong Luo (HXTL) capsule is composed of seven herbs: pea (Leguminosae), Danggui (Apiaceae), Peony (Paeoniaceae), Ligusticum (Apiaceae), Peach kernel (Rosaceae), Carthamus (Compositae), and Rehmannia (Scrophularia). It has been used in China to treat osteonecrosis of the femoral head.¹⁶ The HXTL capsule can improve blood circulation, increase blood flow

throughout the body, reduce swelling, and relieve pain. Our previous study also demonstrated that it could promote osteogenesis by inhibiting the transcriptional expression of lncRNA Miat.¹⁷ However, the effect and mechanism of HXTL capsule in treating PMOP remain unclear. Therefore, in this study, we used network pharmacology-based mechanism prediction, molecular docking, and pharmacological verification to explore its potential treatment of PMOP in ovariectomized mice.

Materials and methods

HXTL capsule

HXTL (approved by the institution under number Z20071224) was clearly introduced in previous studies.^{16,17} It consists of seven types of herbs, which were acquired from the First Affiliated Hospital of Guangzhou University of Chinese Medicine. The botanical names of all herbs mentioned in Table S1 can be found in “The Plant List” (www.theplantlist.org), and their indices align with the criteria set forth in the Chinese Pharmacopoeia (2020).

HXTL decoction preparation

For the *in vitro* experiment, the powder mixture of HXTL was weighed, then boiled in distilled water for 30 m, and centrifuged to obtain the supernatant. The dregs were dried and weighed again to calculate the weight of the dissolved mixture. Finally, the supernatant was concentrated to 5 mg/mL for subsequent experiments.

For the *in vivo* study, a dose of 8 mg/kg/day HXTL decoction was prepared according to the clinical dose and dose translation ratio between humans and mice.¹⁸ The powder mixture of HXTL was weighed and added to distilled water to a concentration of 80 mg/mL for subsequent use.

Analysis of ingredients and target prediction for HXTL capsule

Using the TCMSP database (<https://tcmsp-e.com/tcmsp.php>), we screened the active components of HXTL capsule, excluding *Cajanus cajan*, as previously reported.¹⁹ The ingredients of *Cajanus cajan* were obtained through the Web of Science and PubMed databases using the keywords “pigeon pea leaves”,^{20–24} followed by further screening using SwissADME. The thirteen compounds identified in *Cajanus cajan* are listed in Table S2. Finally, the potential targets of HXTL were predicted using the Swiss Target Prediction Database (<http://swisstargetprediction.ch/>), and protein targets were searched for in the UniProt database (<https://www.uniprot.org/>) to standardize and obtain canonical protein target information.²⁵

Acquiring the disease targets of PMOP

The treatment targets for PMOP therapy were obtained from the GeneCards database (<http://www.genecards.org/>). “Postmenopausal Osteoporosis” was searched under *Homo sapiens* as previously done.²⁶

The common targets of HXTL against PMOP and protein-protein interaction network construction

Using Venny 2.1, we identified the common targets between PMOP and HXTL. We subsequently employed the STRING database to examine co-expression data within the protein-protein interaction (PPI) network of the targeted genes, establishing a threshold interaction score of 0.7. Following this, we uploaded the TSV files into the Cytoscape application (version 3.7.1) to visualize the PPI network. We applied MCODE clustering anal-

ysis to identify relevant functional network modules. Next, we calculated the degree centrality of the co-expression network and extracted subnetworks to identify key nodes in the network using established methods.²⁷

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis

We employed the DAVID database (<https://david.ncicrf.gov/>) to perform GO and KEGG pathway enrichment analyses on all prospective therapeutic targets. This methodology elucidated significant pathways and correlated GO terms spanning the categories of Biological Process, Molecular Function, and Cellular Component.²⁸ We considered pathways and GO entries significant if they had a *P*-value less than 0.05 and retained them for further analysis. We visualized the results of the GO and KEGG enrichment analyses using bioinformatics tools, including signal pathway bar charts and GO category bubble charts. Additionally, we employed Cytoscape v3.7.1 software to visualize the network of active compounds, potential targets, and signal pathways.

Molecular docking

The three-dimensional configuration of the core compound was acquired from the PubChem database. Next, the 3D structure was retrieved from the PDB database (<http://www.rcsb.org/>). Using PyMOL software, water molecules and original ligands were removed from the protein. Then, AutoDock Tool 1.5.7 was employed to add hydrogens, compute charges, and manage non-polar hydrogen bonds. Both the ligand and receptor were saved as PDBQT files before proceeding with molecular docking and visualization.^{29,30}

Cell culture and western blot analysis

RAW264.7 cells were seeded in six-well plates and induced to differentiate into osteoclasts with receptor activator of nuclear factor- κ B ligand (RANKL) (50 ng/mL) in the presence of HXTL extract (5 μ g/mL) for 0, 15, 30, 45, and 60 min, respectively. Total proteins were extracted for subsequent western blot analysis. The primary antibodies used for detection were PI3K antibody (1:1,000, Affinity Biosciences), p-PI3K antibody (1:1,000, Affinity Biosciences), AKT antibody (1:1,000, Affinity Biosciences), phosphorylated AKT (1:1,000, Affinity Biosciences), and β -actin antibody (1:1,000, Santa Cruz). After incubation with the appropriate secondary antibody, protein bands were visualized using an enhanced chemiluminescence detection kit.

In vitro osteoclast differentiation assay

RAW264.7 cells were plated in a 96-well plate at 7×10^3 cells/well and stimulated with 50 ng/mL macrophage colony-stimulating factor and RANKL to differentiate into osteoclasts. Simultaneously, cells were exposed to varying HXTL concentrations (0, 2.5, 5 μ g/mL). The medium was changed every three days, and induction was stopped after six to seven days, upon confirmation of mature osteoclasts. Samples were fixed with paraformaldehyde, washed with phosphate buffer saline, stained with a tartrate-resistant acid phosphatase (TRAP) kit, and imaged using an Olympus inverted microscope. Osteoclast quantification was performed using ImageJ software.

Animal model and micro-computed tomography (micro-CT)

Thirty-two specific pathogen-free (SPF) female C57BL/6J mice aged eight weeks were acquired from the Guangdong Medical Laboratory Animal Center under the license number SCXK, 2022-0002. Housing was provided in the SPF Laboratory Animal Center

at Guangzhou University of Chinese Medicine, adhering to standardized conditions. Throughout the duration of the experiment, the living conditions were meticulously regulated. The mice were randomized into four groups: sham operation (*n* = 8), ovariectomized (OVX) (*n* = 8), OVX with low-dose HXTL (200 μ L by gavage, 8 mg/kg/day) (*n* = 8), and OVX with high-dose HXTL (200 μ L by gavage, 16 mg/kg/day) (*n* = 8). Under anesthesia with pentobarbital sodium, ovaries were removed in the OVX group, while only a small amount of surrounding adipose tissue was removed in the sham group, leaving the ovaries intact. The mice were then treated with HXTL or an equivalent volume of saline for eight weeks. The ratio of bone volume to total volume (BV/TV), trabecular number (Tb. N), and trabecular thickness (Tb. Th) were quantified using CT Analyzer software.

Histological analysis

Bone samples were fixed in 10% formaldehyde for 48 h and then preserved in 70% ethanol. Subsequently, all spinal samples were decalcified, embedded in paraffin, and sectioned into 5-micrometer slices. We then performed TRAP staining (DH0006, LEAGENE, China) according to the kit instructions. Additionally, we detected the expression of osteopontin (OPN) antibody (1:300, Santa Cruz) and phosphorylated AKT (1:300, Affinity Biosciences) in bone tissue using immunohistochemical methods.

Statistical analysis

All data were processed using GraphPad Prism 9.0 software (San Diego, USA), and results are presented as mean $\pm$ standard deviation. Both two-way and one-way analyses of variance were utilized to evaluate differences between the groups at various time points. Statistical significance was established at *P* < 0.05.

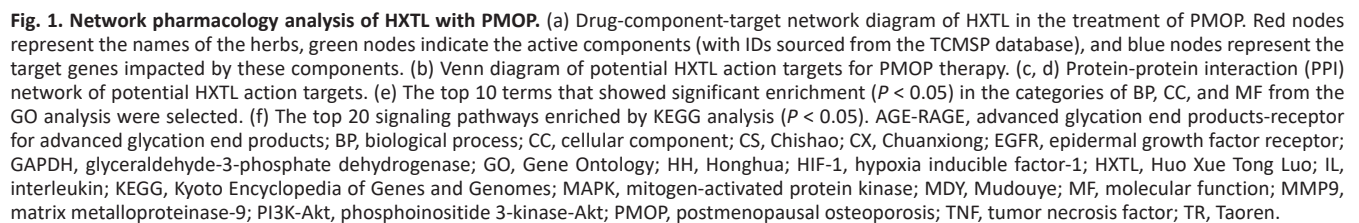
Results

Network pharmacology analysis of HXTL for the treatment of PMOP

In Figure 1a, we constructed a Drug-Compound-Target network using Cytoscape (3.7.0), including components from seven herbs in HXTL, 85 bioactive compounds, and 519 corresponding targets. Following this, we aligned the target genes with PMOP-related genes sourced from GeneCards, identifying 215 common targets, as shown in the Venn diagram (Fig. 1b). We then selected the top 10 genes from the PPI network, ranked by relevance, and visualized by node size and color intensity (Fig. 1c). The PPI results, downloaded from the STRING database, were visualized with Cytoscape (3.7.0), revealing 206 nodes and 3,694 edges (Fig. 1d). The 215 intersecting genes were subjected to GO and KEGG assessments. The GO enrichment analysis specifically highlighted the positive regulation of MAP kinase activity, the MAPK cascade, and responses to estradiol and estrogen response element binding (Fig. 1e). The KEGG pathway analysis indicated that the PI3K-AKT signaling pathway might be the main signaling pathway involved in treating PMOP with HXTL (Fig. 1f).

Molecular docking of the main components of HXTL and pathway-related proteins

Next, the components of HXTL were filtered by their degree values, and four core components (luteolin, beta-sitosterol, stigmasterol, and quercetin) were selected for molecular docking with PI3K/AKT and the key osteoclast transcription gene nuclear factor-activated T cell 1 (NFATc1). The 3D visualization of the molecular



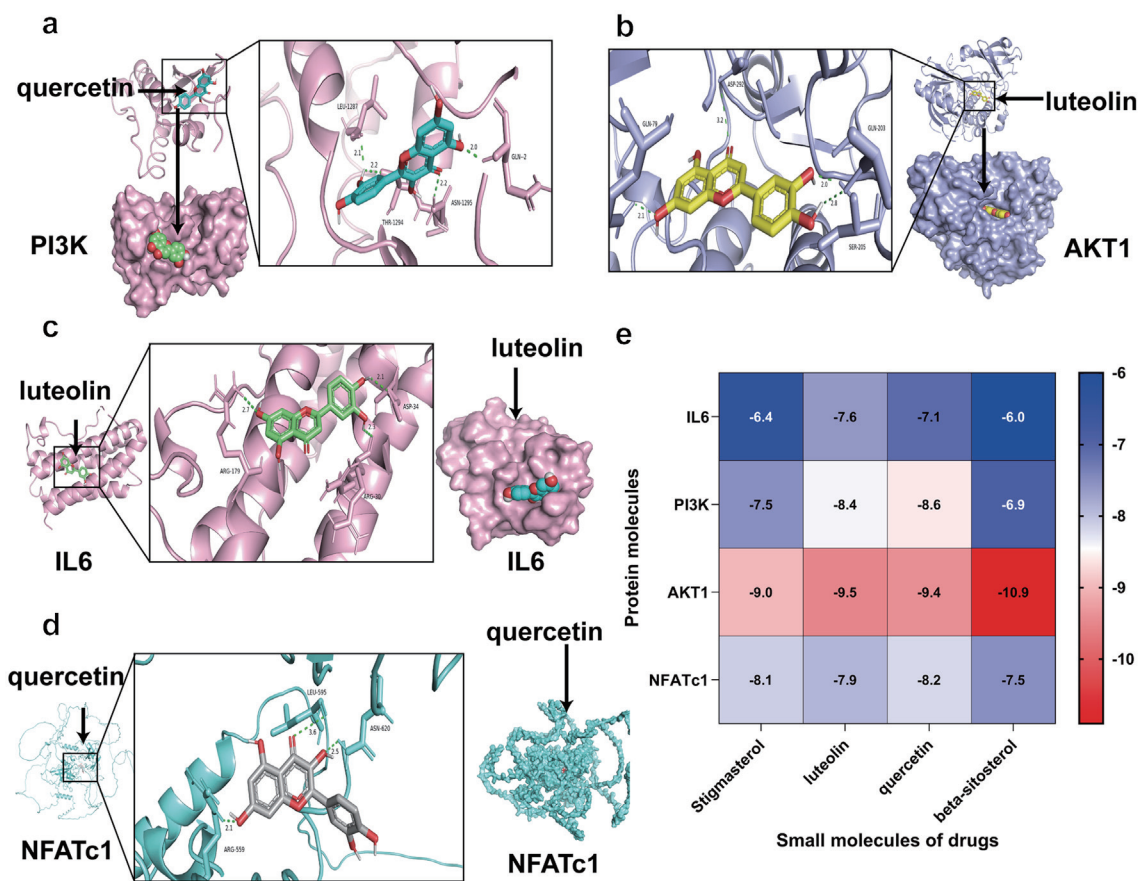


Fig. 2. Molecular docking of the main components of HXTL and pathway-related proteins. (a–c) The 3D visualization of molecular docking modes of HXTL's top four active components (luteolin, beta-sitosterol, stigmasterol, quercetin) and top three HXTL-PMOP targets (IL6, AKT1, PI3K). (d) The docking mode of HXTL with NFATc1. (e) Docking and binding energy of the active ingredient of the core drug combination with the key target protein molecule. AKT1, AKT serine/threonine kinase 1; HXTL, Huo Xue Tong Luo; IL, interleukin; NFATc1, nuclear factor of activated T Cells 1; PMOP, postmenopausal osteoporosis.

docking modes of active HXTL components with PMOP targets (interleukin 6, AKT1, PI3K) and osteoclast marker NFATc1, along with the binding energies, were displayed in Figure 2.

HXTL inhibited osteoclast differentiation *in vitro*

To assess the potential cytotoxic effects of HXTL on the proliferation of RAW264.7 cells, we exposed the cells to different concentrations of HXTL for 48 h, as illustrated in Figure 3a. The Cell Counting Kit-8 (CCK-8) assay results revealed that there was no significant alteration in cytotoxicity within the concentration range of 0 to 5 µg/mL; however, a reduction in cellular viability was observed at 10 µg/mL. Consequently, we chose the highest non-cytotoxic concentration of 5 µg/mL for subsequent cellular experiments. Guided by these findings, we further demonstrated that HXTL could impede the maturation and differentiation of osteoclasts using TRAP staining, as shown in Figure 3b, c.

HXTL inhibited the PI3K-AKT signaling pathway during osteoclastogenesis

We elucidated the mechanism by which HXTL inhibited osteoclastogenesis, specifically the PI3K-AKT signaling pathway, identified through network pharmacology analysis. RAW264.7 cells were treated with RANKL to induce osteoclastogenic differentiation in the presence of HXTL. Western blot analysis showed that

the levels of p-AKT and p-PI3K decreased steadily from 15 to 45 m after HXTL treatment (Fig. 4).

HXTL protected against OVX-induced bone loss

Finally, we established the OVX-induced osteoporosis model to explore HXTL's potential therapeutic effect. The micro-CT analysis of the distal femur revealed significant improvements in bone metrics, such as bone volume fraction (BV/TV), trabecular number, trabecular thickness, and trabecular separation. The improvements were notable in both the high and low concentrations of HXTL, with the 10 mg/kg dosage showing the best results, as shown in Figure 5. HE staining results demonstrated a significant decrease in bone mass in the model group compared to the sham group, while the HXTL group demonstrated a significant increase in both the number and volume of trabecular bone. TRAP staining showed a decrease in the number of osteoclasts after HXTL treatment (Fig. 6), suggesting that HXTL inhibits osteoclast formation *in vivo*. Moreover, the levels of OPN and p-AKT were significantly increased by HXTL capsule treatment, as demonstrated by immunohistochemical assays (Fig. 6).

Discussion

PMOP has become a severe public health problem worldwide

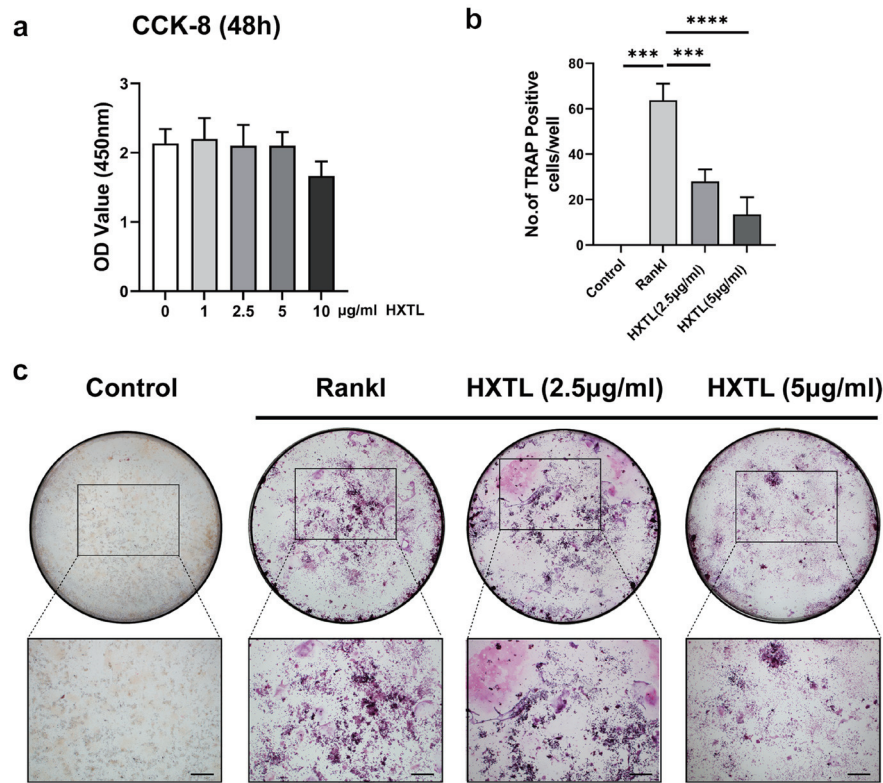


Fig. 3. HXTL suppressed RANKL-mediated osteoclast differentiation. (a) The CCK-8 assay was employed to evaluate the impact of various HXTL concentrations on RAW264.7 cell viability. (b, c) TRAP staining was utilized to demonstrate the effects of different HXTL concentrations (0, 2.5, and 5 μg/mL) on osteoclast differentiation, along with a quantitative analysis. The scale bar represents 200 μm. Data are presented as mean ± SD, with each group consisting of three samples (n = 3). Significance levels are indicated as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. CCK-8, Cell Counting Kit-8; HXTL, Huo Xue Tong Luo; OD, optical density; RANKL, receptor activator of nuclear factor-κB ligand; SD, standard deviation; TRAP, Tartrate resistant acid phosphatase.

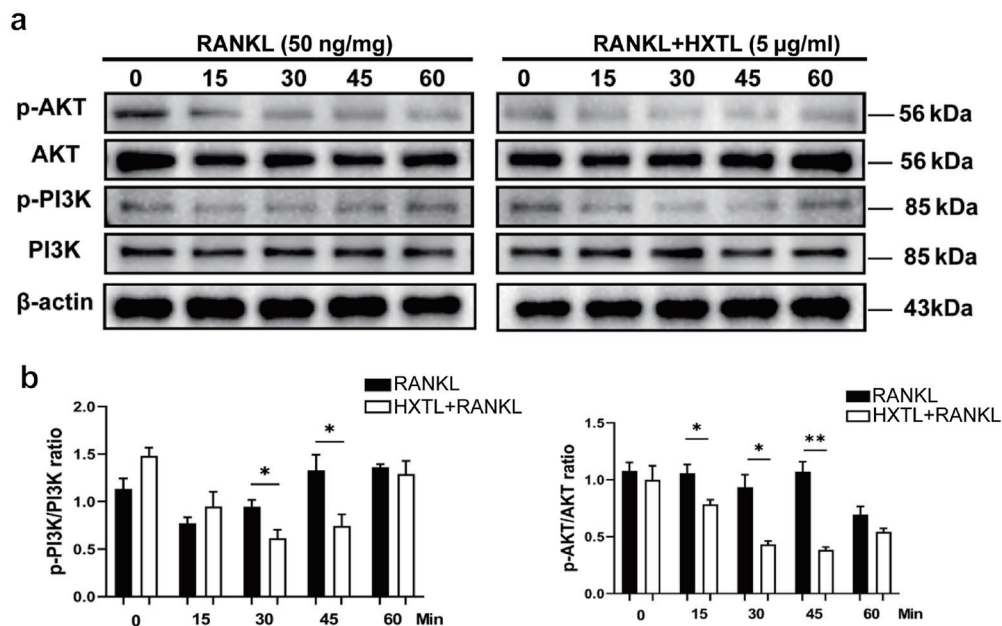


Fig. 4. HXTL inhibited the PI3K-AKT signaling pathway during osteoclastogenesis in RAW264.7 cells. (a, b) Western blot analysis was performed at different time points (0, 15, 30, 45, 60 min) to detect phosphorylated PI3K and AKT in the presence of RANKL and RANKL+HXTL. The results are shown as the mean ± SD. * $P < 0.05$, ** $P < 0.01$. HXTL, Huo Xue Tong Luo; PI3K-AKT, phosphoinositide 3-kinase-Akt; RANKL, receptor activator of nuclear factor-κB ligand; SD, standard deviation.

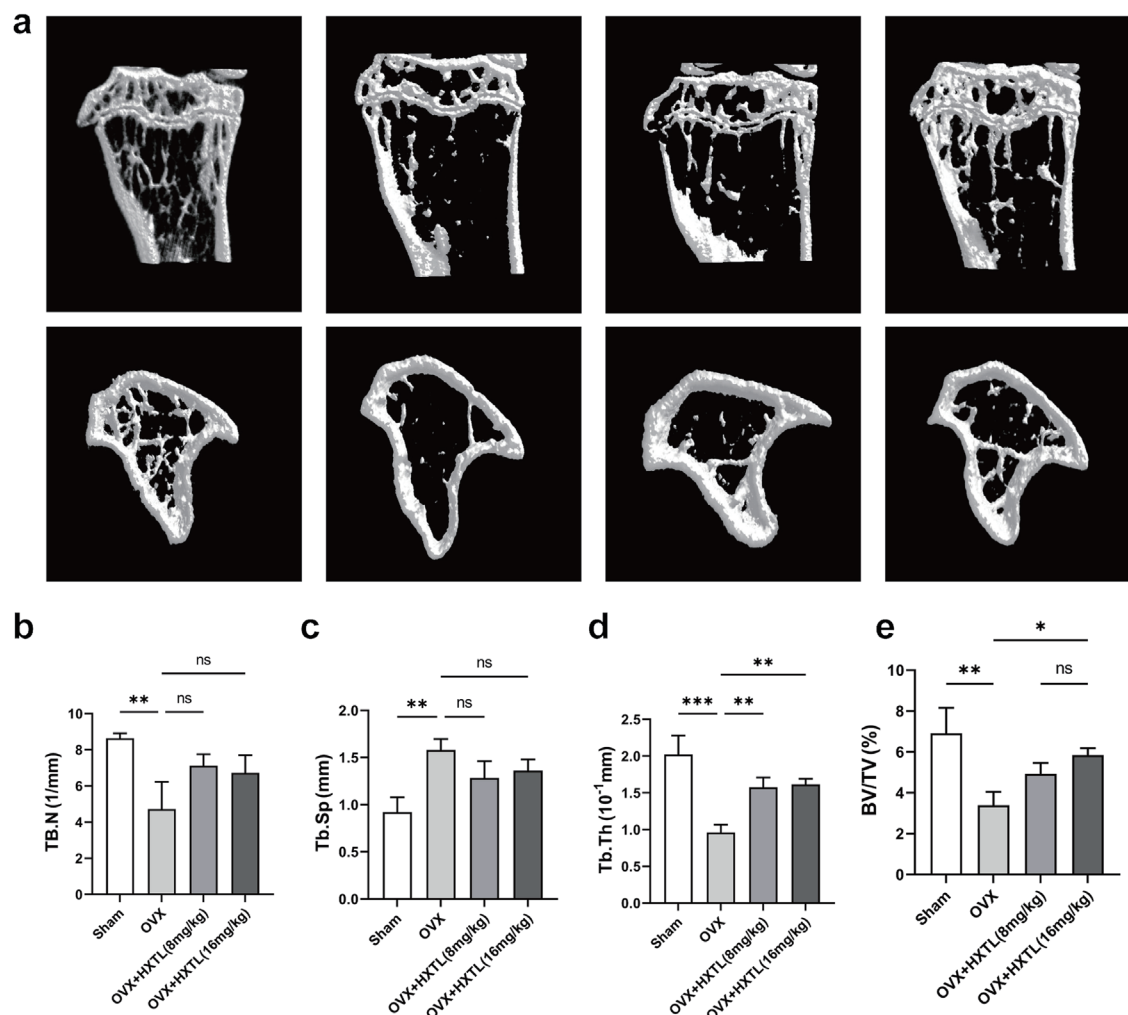


Fig. 5. HXTL attenuated bone mass loss in the OVX model. (a) Three-dimensional CT images of the distal femur are presented. (b-e) The analysis of bone parameters, including BV/TV, Tb.N, Tb.Th, and Tb.Sp, was conducted in the region below the growth plate of the distal femur ($n = 6$ per analysis). Data are expressed as mean $\pm$ SD, with $n = 8$ animals per group. Significance levels relative to the OVX group are denoted as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. BV/TV, bonevolume fraction; HXTL, Huo Xue Tong Luo; OVX, ovariectomized; SD, standard deviation; TV, total volume.

with the progress of the aging population.³¹ Due to the side effects of current anti-osteoporosis drugs, it is urgent to develop a more effective and safer treatment strategy.³² Previous studies have shown that the HXTL capsule promotes the osteogenic differentiation of human bone marrow mesenchymal stem cells (MSCs) and prevents the progression of asymptomatic osteonecrosis of the femoral head.^{16,17} However, its effect on osteoporosis has not been investigated. In this study, we explored the anti-osteoporosis effect and the potential mechanisms of the HXTL capsule through network pharmacology-based mechanism prediction and pharmacological validation. We found that HXTL prevented bone loss induced by osteoclast formation by inhibiting the PI3K-AKT signaling pathway.

Bone homeostasis requires a dynamic balance between bone formation and bone resorption. Osteoporosis occurs when osteoblast-mediated bone formation is slower than bone resorption directed by osteoclasts.³³ Thus, the abnormal proliferation and apoptosis of osteoblasts and osteoclasts directly result in osteoporosis. NFATc1 is a crucial transcription factor in the differentiation

process of osteoclasts.^{34,35} The activation of NFATc1 depends on the RANKL-RANK signaling pathway, which plays a pivotal role in the induced differentiation of osteoclast precursors.³⁶ NFATc1 directly regulates several genes related to osteoclast functionality, including those encoding degradative enzymes and proteins associated with bone absorption.³⁷ OPN, a major secreted phosphoprotein in bone, plays an important role in bone metabolism and homeostasis. OPN can stimulate the proliferation and calcification of osteoblasts and mediate bone metabolism caused by mechanical stress.³⁸ It has been generally used as a marker of osteogenesis. The higher level of OPN in HXTL capsule-treated mice provides strong evidence for its role in PMOP prevention.

Numerous studies have proven that the PI3K-AKT signaling pathway is involved in osteoclast differentiation. It is well known that RANKL is indispensable for the differentiation of hematopoietic precursor cells into osteoclasts. The binding of RANKL to its receptor RANK activates the PI3K-AKT pathway through Src kinase, as genetic deletion of c-Src inhibits

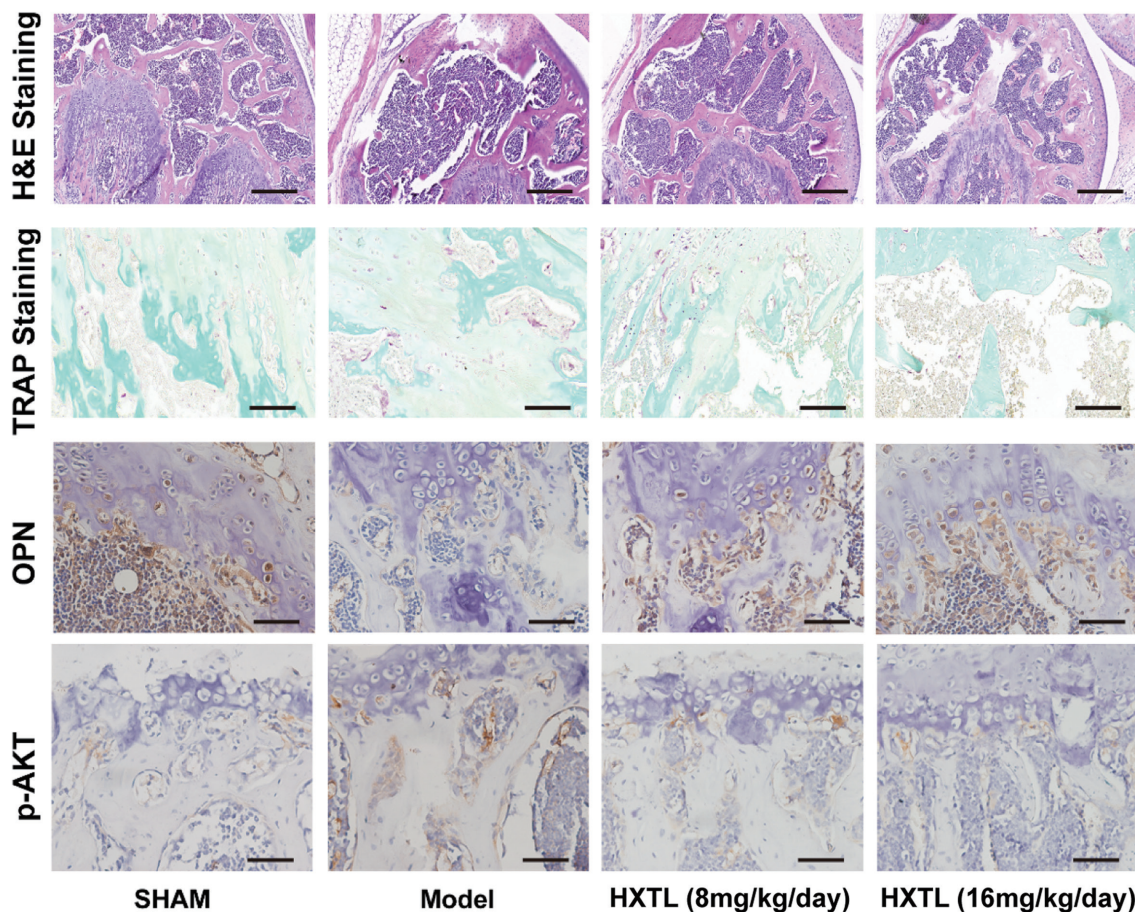


Fig. 6. Histological analysis. The distal femur of each group was sectioned and subjected to HE staining, TRAP staining, and immunohistochemical detection with specific antibodies. Scale bar = 50 μ m. HE, hematoxylin-eosin staining; HXTL, Huo Xue Tong Luo; OPN, osteopontin; p-AKT, phosphorylated AKT serine/threonine kinase 1; SHAM, Sham operation; TRAP, tartrate resistant acid phosphatase.

RANKL-mediated AKT activation.³⁹ In addition, the PI3K inhibitor LY294002 has also been demonstrated to have an inhibitory effect on osteoclast formation.⁴⁰ Our results clearly showed that HXTL could inhibit the PI3K-AKT signaling pathway. Furthermore, our *in vivo* experiments also confirmed the inhibitory effect of HXTL on phosphorylated AKT. Our previous study showed that the HXTL capsule can promote the osteogenesis of MSCs,¹⁷ and it also inhibited the formation of osteoclasts, as demonstrated in this study, which clearly explains its preventive effect on PMOP.

The active compound is an important factor in explaining the effects of herbs. There are seven herbs in the formulation of the HXTL capsule, including cajan leaf, tails of Angelica, red peony root, Ligusticum wallichii, peach kernel, Carthamus tinctorius, and Radix Rehmanniae preparata. Some active compounds have been reported to regulate osteogenesis or osteoclastogenesis and prevent bone loss in animal models of osteoporosis. For example, genistein, a non-steroidal phytoestrogen found in HXTL capsule and various herbs, can potentially replace endogenous estrogens in postmenopausal women.⁴¹ Many studies have demonstrated that genistein not only inhibited osteoclastogenesis but also increased osteoblastogenesis.^{42,43} Vanillic acid, a phenolic compound in HXTL capsule, could also promote the osteogenic differentiation of MSCs and inhibit PMOP in rats.^{44,45} Shintaro Onishi *et al.* have

reported the inhibitory effect of luteolin and its derivatives on osteoclast differentiation.⁴⁶ These compounds are the material basis of the HXTL capsule's bioactivity. The limitation of this study is that the main active compound regulating the PI3K-AKT signaling pathway remains unidentified.

Conclusions

Taken together, this study demonstrates that HXTL capsule treatment effectively mitigates OVX-induced bone loss by inhibiting the PI3K-AKT signaling pathway, which expands the application field of HXTL capsule in treating bone diseases.

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Conflict of interest

One of the authors, LX, has been an Associate Editor of *Future Integrative Medicine* since October 2021. The authors have no other conflict of interest to note.

Author contributions

Experiment performance (QZ), drafting of the manuscript (QZ, FC), validation (FC, HL, RH, LH, QW), formal analysis (HL, RH, LH, QW), supervision, resources, and conceptualization (LX, YL). All authors have approved the final version and publication of the manuscript.

Ethical statement

This study was carried out in accordance with ethical standards of Laboratory Animals of Guangzhou University of Chinese Medicine. The protocol was approved by the Animal Experiment Ethics Committee of Guangzhou University of Chinese Medicine (Approval Number: 20230417003).

Data sharing statement

The data of this study are available from the corresponding author upon reasonable request.

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