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Protective effects and mechanism of chrysophanol against age-related osteoporosis in rats

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ABSTRACT

Objective(s): This study aimed to investigate the protective effect and underlying mechanism of chrysophanol (CPH) on senile osteoporosis (OP) in rats.

Materials and Methods: Twenty-four-month-old rats were divided into an aged OP model group and low- (10 mg/kg), medium- (20 mg/kg), and high-dose (50 mg/kg) CPH groups, with eight-month-old rats used as the control group. After 60 days of oral CPH administration, the bone mineral density (BMD), bone volume fraction (BV/TV), trabecular number (TbN), and trabecular separation (TbSp) of the femur were analysed using Micro-CT. Serum levels of bone formation markers (alkaline phosphatase, ALP; osteocalcin, OC) and bone resorption markers (type I collagen crosslinked N-telopeptide, NTX; tartrate-resistant acid phosphatase, TRACP) were determined by ELISA. Pathological changes in the femur were observed via H&E staining, and the expression of p-AKT and p-mTOR proteins in bone marrow was detected by western blotting.

Results: Compared with those in the control group, the bone formation marker levels, BMD, BV/TV, and TbN in the model group significantly decreased, whereas the bone resorption marker levels, TbSp, and expression levels of p-AKT and p-mTOR significantly increased, with obvious bone structure destruction. CPH treatment reversed these changes in a dose dependent manner, significantly enhanced bone formation, inhibited bone resorption, improved bone density and microstructure, and down-regulated the expression of p-AKT and p-mTOR.

Conclusion: CPH can effectively improve bone density and microstructure in aged osteoporotic rats, and its bone-protective effect may be related to the inhibition of the PI3K/AKT/mTOR signaling pathway.

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Introduction

Osteoporosis is a systemic bone metabolism disorder characterized by impaired bone mass, bone strength, and microarchitecture that primarily affects elderly individuals (1). Osteoporosis currently affects approximately 500 million people worldwide and continues to increase as the population ages (2). The prevalence of osteoporosis in the Chinese population ≥ 60 years of age is reported to be approximately 37.7%, and the incidence increases with age, which severely affects daily activities and quality of life (3). Bone mass is maintained by a dynamic balance between bone resorption and formation, with osteoclasts, osteoblasts, and osteocytes playing critical roles in bone homeostasis. This complexity is regulated by multiple cellular and molecular mechanisms (4). Decreased osteogenic activity in osteoblasts and enhanced osteoclast resorption activity in elderly individuals are key pathological mechanisms leading to osteoporosis. Therefore, restoring the balance of bone metabolism through targeted therapeutic intervention is essential for the treatment of osteoporosis.

Studies have shown that rhubarb has multiple pharmacological effects, such as antioxidant, anti-inflammatory, and antibacterial effects, and can regulate bone metabolism (5). Chrysophanol (1, 8-dihydroxy-3-methyl-anthraquinone, also known as chrysophanic acid, CAS No.: 481-74-3) is a natural anthraquinone derivative widely present in various traditional Chinese medicines, such as rhubarb, cassia seed, *Polygonum multiflorum*, aloe vera and senna leaf, and has been confirmed to be one of the key active components that exerts pharmacological effects (6-7). Chrysophanol, which has been extensively studied, has shown potential utility in the prevention and treatment of various diseases, such as cancer, atherosclerosis, asthma, diabetes, atopic dermatitis, and osteoarthritis (8-10). These studies further revealed that chrysophanol could exert its biological activity through multiple molecular mechanisms, and recent studies revealed that chrysophanol could stimulate bone growth by promoting osteogenic

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differentiation through the activation of the AMPK/Smad1/5/9 signaling pathway (11). The detailed molecular mechanisms underlying its effects on osteoporosis development and progression remain unclear. However, the molecular mechanism through which chrysophanol affects the development and progression of osteoporosis remains to be further elucidated.

The PI3K/AKT/mTOR signaling pathway is a key pathway that regulates cellular processes and is widely involved in the pathophysiology of various diseases, including osteoporosis (12-14). Zhang *et al.* revealed that the PI3K/AKT/mTOR signaling pathway regulates the proliferation, migration and invasion of osteoblasts (15). In addition, Yang *et al.* reported that the inhibition of the PI3K/AKT/mTOR signaling pathway enhanced mitophagy and thereby protected osteoblasts (16). In addition, chrysophanol, an inhibitor of this pathway, potentially improves neurological dysfunction caused by intracerebral hemorrhage (17). On the basis of the above findings and their therapeutic potential, the aim of this study was to investigate whether chrysophanol exerts a protective effect by regulating the PI3K/AKT/mTOR signaling pathway and to elucidate its related mechanisms.

Materials and Methods

Experimental animals

Twenty-four Sprague-Dawley (SD) rats (672±73 g) and six 6-month-old SD rats (651±59 g) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd., with the production licence number SCXK (Hu) 2022-0004. All experimental procedures were approved by the Institutional Animal Ethics Committee (IACUC) of the Second Military Medical University (Protocol Number: 2023-0524-1) and performed in accordance with the Guide for the Care and Use of Laboratory Animals. All experimental animals were housed in a temperature-controlled environment (23±2 ˚C) in an SPF-grade environment alternating 12 hr of light and dark every day with free access to food and water.

Main instruments and reagents

A Viva CT40 Micro-CT instrument (SCANCO Medical AG, Switzerland), electrophoresis instrument and electrotransfer instrument (Bio-Rad, USA), and microplate reader (Thermo Fisher, USA) were used. Rhubarb (Shanghai Aladdin Biochemical Technology Co., Ltd.); ALP and OC ELISA kits (Shanghai Youkewei Biotechnology Co. Ltd.), NTX and TRACP ELISA kits (RampD, USA), H&E staining kits (Nanjing Jiancheng Bioengineering Institute), p-Akt, Akt, p-mTOR, mTOR (all purchased from Abcam, UK), and GAPDH (Cell Signaling Technology, USA) primary antibodies, horseradish peroxidase-labelled secondary antibodies (Abcam, UK), and enhanced chemiluminescence kits (Millipore, USA) were also used.

Establishment of the experimental animal model

A model of senile osteoporosis was developed using naturally ageing male rats based on previously established methods (18). Naturally aged 24-month-old SD rats were used as an aged osteoporosis model. Rats in this age group are equivalent to humans who are 65-70 years old, and the bone metabolism and bone structure changes in rats naturally mimic the pathological process of senile osteoporosis in humans.

Animal grouping and experimental design

Twenty-four 24-month-old SD rats were randomly divided into 4 groups (n=6) and treated with chrysophanol (10, 20 and 50 mg/kg) according to a previous study (19). The groups were as follows: osteoporosis model group, model+low-dose chrysophanol group (L-CPH, 10 mg/kg), model+medium-dose chrysophanol group (M-CPH, 20 mg/kg), and model+high-dose chrysophanol group (H-CPH, 50 mg/kg). Each group was administered daily by gavage for 60 days. In addition, six 8-month-old healthy SD rats were used as the control group (this age in rats is approximately equivalent to 25-year-old humans) and treated with normal saline. At the end of the experiment, the rats were sacrificed, and serum and femur samples were collected for subsequent testing.

Bone morphometric indices

After the rats were sacrificed, their femurs were removed, the excess muscle tissue was removed and rinsed in saline to remove surface blood, and the tissue was fixed in 4% neutral paraformaldehyde. Then, micro-CT scanning and BMD were performed, and the following trabecular parameters were calculated: BV/TV, TbN, and TbSp.

Histological examination

Following decalcification of the femoral tissues, the samples were sequentially dehydrated with graded ethanol, cleared in xylene, and subsequently embedded in paraffin, after which 5-μm-thick sections were prepared. After deparaffinization with xylene and rehydration with graded ethanol, the sections were stained according to a standard H&E staining procedure, and finally, the pathological changes in the femoral tissue samples were observed and photographed under a light microscope (50×).

Serum biochemical analysis

Blood samples were collected from all experimental groups and centrifuged to obtain serum. The levels of the bone metabolism markers ALP, OC, NTX, and TRACP were measured according to the ELISA kit instructions. The absorbance was measured at 450 nm using a microplate reader. Samples were then calculated from a standard curve established for each marker.

Western blot analysis

Bone marrow tissue proteins were extracted and lysed using RIPA lysis buffer containing 1% protease inhibitors, and protein concentrations were determined using the Bradford method. The proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, blocked with 5% skim milk for 1 hr at room temperature, and subsequently incubated with primary antibodies overnight at 4 ˚C. The following day, the membranes were incubated with the corresponding secondary antibodies for 1 hr. After ECL development, band grayscale analysis was performed using ImageJ software. GAPDH was used as an internal reference.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA, USA). The data are presented as the mean±standard deviation (mean±SD), and one-way analysis of variance (one-way ANOVA) was used

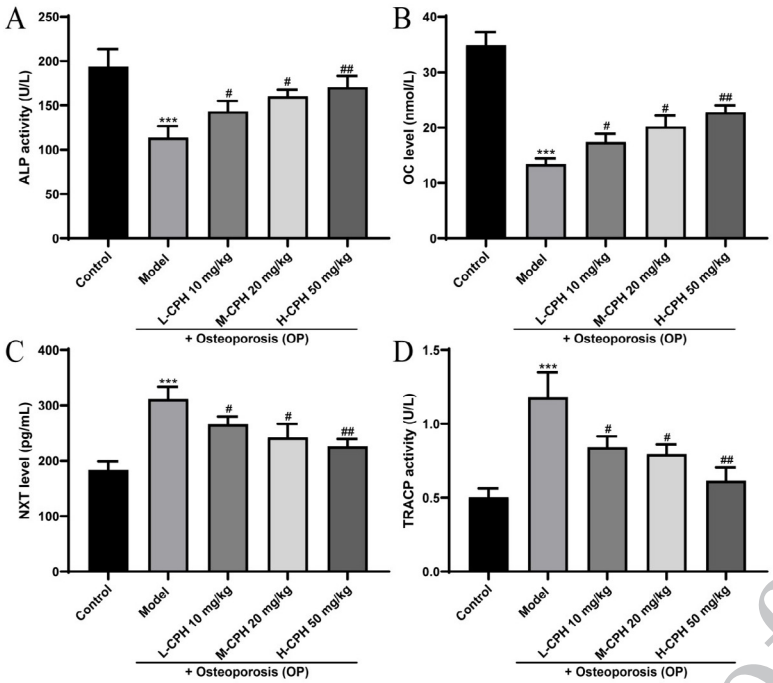

Figure 1. EEffect of chrysophanol (CPH) on bone metabolism markers in osteoporotic rats
A. Serum alkaline phosphatase (ALP) concentration; B. Serum osteocalcin (OC) concentration; C. Serum type I collagen crosslinked N-telopeptide (NTX) concentration; D. Serum tartrate-resistant acid phosphatase(TRACP) concentration. ***P<0.001 compared with the control group; #P<0.05, ##P<0.01 compared with the Model group

to compare multiple groups; if the results were significant, the Tukey test was used for pairwise comparisons. P<0.05 was considered to indicate statistical significance.

Results

Effects of CPH on bone metabolic markers in osteoporotic rats

Compared with those in the control group, the levels of the bone formation markers ALP and OC in the serum of osteoporotic model rats significantly decreased (P<0.01), while the levels of the bone resorption markers NTX and TRACP significantly increased (P<0.01). After intervention with low, medium, and high doses of CPH, compared with those in the model group, the serum ALP and OC levels in each treatment group significantly increased (P<0.05), while the NTX and TRACP levels significantly decreased (P<0.05). The results are shown in Figure 1.

Effects of CPH on bone morphometric indicators in osteoporotic rats

Micro-CT reconstructed images (Figure 2A) revealed that compared with those in the control group, the number of trabeculae in the osteoporosis model group was significantly lower; however, after intervention with low, medium, and high doses of CPH, the number of trabeculae in each treatment group significantly increased compared with that in the model group. Further quantitative analysis revealed that compared with those in the control group, the femoral BMD (Figure 2B), BV/TV (Figure 2C) and TbN (Figure 2D) were significantly lower, and the TbSp (Figure 2E) was significantly greater in the model group (all P<0.01). Compared with the model group, the low-, medium-, and high-dose CPH treatment groups exhibited significant increases in femoral BMD, BV/TV, and TbN and a decrease in TbSp (all P<0.05).

Effects of CPH on histopathological findings in the femurs of osteoporotic rats

H&E staining results revealed (Figure 3) that compared with those in the control group, the number of femoral trabeculae was significantly reduced, the spacing was widened, the structure was severely damaged, and more cavity formation was observed in the bone marrow cavity in the osteoporosis model group; however, after intervention with low, medium, and high doses of CPH, the bone structure

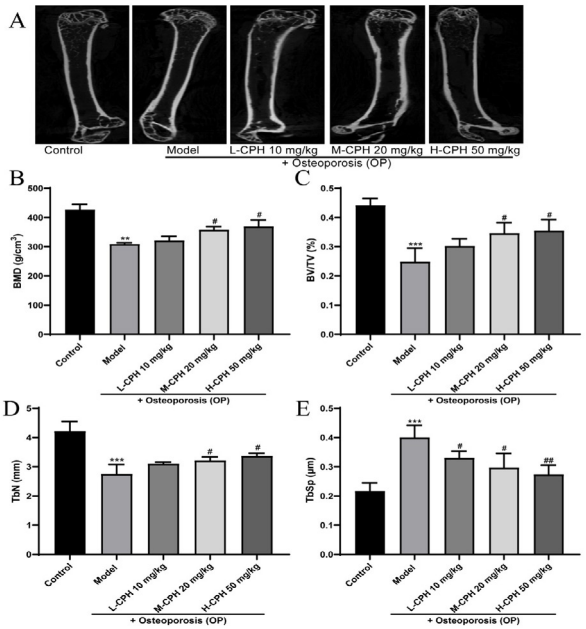

Figure 2. EEffects of chrysophanol (CPH) on morphological parameters in osteoporotic rats
A. Micro-CT images; B. Bone Mineral Density (BMD); C. Bone Volume/Tissue Volume (BV/TV); D. Trabecular Number (TbN); E. Trabecular Separation (TbSp). **P<0.01, ***P<0.001 compared with the control group; #P<0.05, ##P<0.01 compared with the Model group

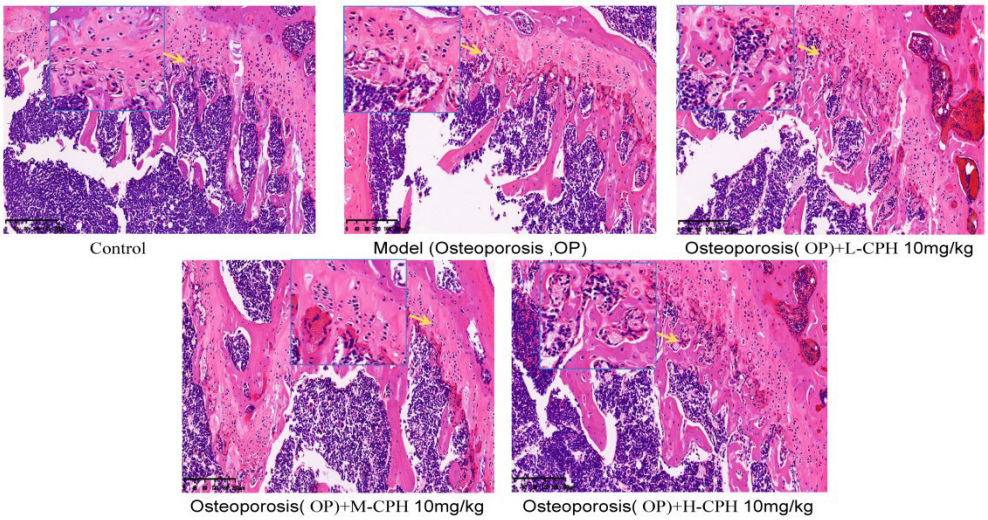

Figure 3. Effect of chrysophanol (CPH) on histopathological findings of bone and femur in osteoporotic rats

was significantly improved in each treatment group, and the trabecular arrangement, spacing, and thickness were restored compared with those in the model group.

Effect of CPH on the PI3K/AKT/mTOR signaling pathway in the bone marrow of osteoporotic rats

Compared with those in the control group, the expression levels of p-AKT and p-mTOR proteins in the bone marrow of rats in the osteoporosis model group significantly increased (P<0.001); however, after intervention with low, medium, and high doses of CPH, the expression of p-AKT and p-mTOR significantly decreased in each treatment group (P<0.05). The results are shown in Figure 4.

Discussion

In this study, we simulated age-related bone loss in 24-month-old rats and reported that CPH treatment could regulate bone metabolism, increase the expression of bone formation markers (ALP and OC) and decrease the expression of bone resorption markers (NTX and TRACP). Moreover, CPH significantly improved bone mineral density and bone microstructure deterioration caused by osteoporosis, increased trabecular thickness, and reduced bone spacing. Molecular mechanism analysis revealed that CPH suppressed the expression of p-AKT and p-mTOR proteins in the femoral bone marrow.

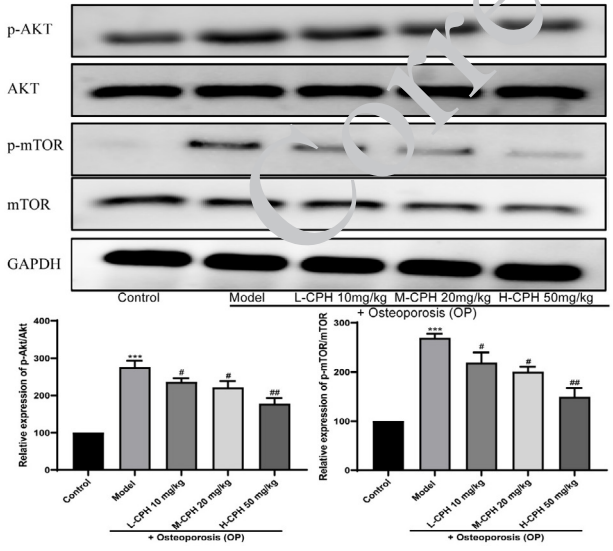

Figure 4. Effect of chrysophanol (CPH) on thePhosphoinositide 3-kinase/ Protein Kinase B/Mammalian Target of Rapamycin (PI3K/AKT/mTOR) signalling pathway in the bone marrow of osteoporotic rats
***P<0.001 compared with the control group; #P<0.05, ##P<0.01 compared with the Model group

Osteoporosis, characterized by osteopenia, compromised bone microarchitecture, increased bone fragility, and increased risk of fractures, is a major public health concern affecting the quality of life of older adults (20). In this study, 24-month-old rats were used to simulate senile osteoporosis, in which osteoblasts and osteoclasts dominate the balance of bone remodelling (21). ALP and OC are key indicators of bone formation, and NTX and TRACP, which are markers of bone resorption, reflect osteogenic and osteoclastic activity, respectively (22). CPH treatment significantly increased serum ALP and OC levels and decreased NTX and TRACP levels, suggesting that it could improve bone metabolism balance by promoting bone formation and inhibiting bone resorption.

Bone mineral density is a core indicator for assessing bone quality and strength and for diagnosing osteoporosis and fracture risk, whereas bone microstructure is a critical parameter for predicting bone loss and structural degeneration (23). In this study, CPH significantly improved bone mineral density and bone microstructural parameters (increased BV/TV and TbN and decreased TbSp), which were verified histologically, consistently indicating significant bone protection.

The PI3K/AKT/mTOR signaling pathway normally orchestrates the physiological functions of osteoblasts and osteoclasts and regulates their activity and survival by acting on specific target genes (24). Activation of the PI3K/AKT signaling pathway by pathological factors can affect the formation, differentiation and function of osteogenesis and osteoclasts, thereby regulating bone mass and strength (25). This pathway plays a key role in maintaining the homeostasis of bone tissue under physiological stimuli and pathological conditions by regulating complex molecular mechanisms, such as cell survival, protein synthesis and metabolism, and may be a therapeutic target for osteoporosis (26). In this study, CPH significantly inhibited the expression of p-AKT

and p-mTOR proteins in this pathway, and these results were consistent with those of previous studies (18); that is, chrysophanol could act as a PI3K/AKT/mTOR signaling pathway inhibitor. These findings suggest that the ability of CPH to improve osteoporosis may be achieved in part by inhibiting this hyperactive signaling pathway.

Conclusion

Chrysophanol can increase bone mineral density, improve bone microarchitecture and promote bone formation in OP aged rats, and its mechanism may involve the regulation of the PI3K/AKT/mTOR signaling pathway.

Acknowledgment

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Ethics Approval and Consent to Participate

The study was conducted in accordance with the Declaration of Helsinki. The research protocol was approved by the Ethics Committee of Experimental Animal Ethics Committee of the Second Military Medical University (No.2310-D-42), and all of the participants provided signed informed consent.

Data Availability Statement

All experimental data and resources utilized in the current investigation are available from the corresponding author upon appropriate request.

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Authors' Contributions

FS S and DG C designed the research study. Q L and CX Z performed the research. LX M, XY Y, and G Z analyzed the data. Q L and CX Z wrote the manuscript. FS S and DG C jointly reviewed and revised the manuscript and finally approved the version to be published. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Declaration

We have not used any AI tools or technologies to prepare this manuscript.

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