Original Article

Zhuanggu Zhitong Capsule alleviates postmenopausal osteoporosis in ovariectomized rats by regulating autophagy through AMPK/mTOR signaling pathway

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Background: Postmenopausal osteoporosis (PMOP) is the most common primary osteoporosis, which is prone to fractures and affect the health and quality of life of the elderly and even shorten their lifetime. Traditional Chinese medicine can not only effectively improve osteoporosis and reduce fracture rate, but also have tonifying and analgesic effects. The purpose of this study was to investigate the effects of Zhuanggu Zhitong (ZGZT) Capsule on autophagy related genes and proteins in PMOP rats, so as to elucidate the molecular mechanism of tonifying deficiency and regulating stasis in the treatment of osteoporosis and analgesia.

Methods: The PMOP rat model was established by bilateral oophorectomy, and then the rats were randomly divided into control group, PMOP group, PMOP + ZGZT group and PMOP + E_2 group. The changes of mechanical pain threshold of rats were detected by von Frey filaments, and the changes of mechanical pain threshold of rats in each group were compared. Computed tomography (CT) and dual-energy X-ray were used to measure the bone mineral density of lumbar bone tissue. Enzyme-linked immunosorbent assay (ELISA) and tartrate-resistant acid phosphatase (TRAP) staining were used to detect inflammatory factors and bone metabolism related indicators. Hematoxylin-eosin (HE) staining was used to observe the tissue morphology of lumbar vertebra tissue. Western blot (WB) and quantitative polymerase chain reaction (qPCR) were used to detect AMPK/mTOR pathway- and autophagy-related factor expression.

Results: ZGZT can effectively restore the bone mineral density (BMD) of PMOP rats, improve the microstructure of lumbar vertebra of PMOP rats, restore the balance of bone metabolism, promote the expression of AMPK and autophagy related factors, inhibit the expression of mTOR and the release of inflammatory factors, and increase the mechanical pain threshold of PMOP rats, so as to effectively improve osteoporosis and relieving osteoporosis pain in PMOP rats.

Conclusions: ZGZT affects autophagy by regulating AMPK/mTOR pathway, restores the homeostasis of bone metabolism and inhibits the release of inflammatory factors. Moreover, the regulation of feedback pathways between bone metabolism and inflammatory factors finally plays the role of "bone strengthening" and "pain relieving". ZGZT may be a new treatment for PMOP and relieving osteoporotic pain.

Keywords: Adenosine 5'-monophosphate-activated protein kinase (AMPK); mammalian target of rapamycin (mTOR); autophagy; postmenopausal osteoporosis (PMOP); inflammatory factor

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Introduction

Postmenopausal osteoporosis (PMOP) is the most common type of osteoporosis (OP). According to statistics, the prevalence of osteoporosis in women over 50 years old is as high as over 32% (1). Fracture and osteoporotic pain are the most direct and obvious symptoms of OP patients, as well as the main reasons for decreased quality of life and medical treatment of OP patients. Fracture was positively correlated with the incidence of pain, at the same time, the PMOP patients were prone to sleep disorders, anxiety, depression, irritability, autism and other psychiatric symptoms. These symptoms cause obstacles to patients' life, work, social activities and other social activities, seriously affect the quality of life of patients, and bring mental and physical pain to PMOP patients. The treatment of PMOP is a severe challenge for clinicians. At present, although there are many western medicines for PMOP treatment, but it is found that long-term use of western medicine has some disadvantages, such as high side effects, high price, fracture probability is still high, and the effect on improving pain, fatigue and other clinical complications is not obvious. At present, it has become a trend to choose Chinese herbal medicines with low toxicity and side effects, reasonable price, address both symptoms and root causes and can be taken for a long time in the treatment of PMOP. In addition, some traditional Chinese medicines can not only effectively improve osteoporosis, but also have good systemic nursing and analgesic effects, which is also the unique advantage of PMOP treatment.

Traditional Chinese medicine (TCM) scholars believe that "deficiency" and "stasis" are the main pathogenesis of PMOP patients. The guidelines for TCM diagnosis and treatment of postmenopausal osteoporosis (bone impotence) are also pointed out that the kidney deficiency, spleen deficiency and blood stasis are the fundamental causes of PMOP, and blood stasis is the fundamental source of PMOP pain symptoms (1). In TCM treatment of PMOP, the methods of tonifying kidney, invigorating spleen and regulating blood stasis are used. Zhuanggu Zhitong (ZGZT) is one of the experiential prescriptions for effective prevention and treatment of PMOP under the guidance of the theory of tonifying deficiency and regulating blood stasis, and it is recommended by the Guidelines for TCM Diagnosis and Treatment of postmenopausal osteoporosis, consisting of fructus psoraleae, epimedium, wolfberry fruit, etc. A study has shown that ZGZT can also be used in the clinical treatment of sarcopenia, and can effectively promote the formation of osteoblasts and inhibit the differentiation of stem cells into osteoclasts in the treatment of osteoporosis (2). A study has shown that ZGZT can significantly improve bone mineral density in primary osteoporosis and relieve symptoms of low back pain, but the mechanism of action remains unclear (3).

The purpose of this study was to investigate the effects of ZGZT on PMOP rats and its mechanism. In the early stage, the research group conducted research and analysis on all relevant targets of PMOP, corresponding targets of ZGZT bioactive ingredients, cross targets and signal pathways of ZGZT treatment of PMOP through network pharmacology. KEGG pathway enrichment analysis showed that estrogen signaling pathway, TNF signaling pathway, IL-17 signaling pathway and mTOR signaling pathway had significant enrichment significance. "mammalian target of rapamycin (mTOR)" genes were found in both ZGZT and PMOP crossover genes in the main KEGG enrichment results, suggesting that the mTOR signaling pathway may be involved in the physiological and pathological process of ZGZT treatment of PMOP. Therefore, its molecular mechanism may be closely related to the mTOR signaling pathway.

Postmenopausal osteoporosis is associated with estrogen deficiency, autophagy imbalance, increased apoptosis, increased reactive oxygen species and other factors (4). Recent studies have found that the occurrence of PMOP is related to autophagy imbalance, autophagy and autophagy related proteins play a very important role in the homeostasis of bone metabolism (5,6). Currently, autophagy is an important direction of OP research, in which activated protein kinase (AMPK)/mTOR signaling pathway mediated autophagy plays an important role in the regulation of bone metabolism homeostasis, and is one of the main convergence points of various factors.

Adenosine 5'-monophosphate (AMP)-AMPK is an energy receptor in cells with the main role of maintaining the energy balance in cells. When the intracellular AMP/

adenosine triphosphate (ATP) ratio is increased, AMPK is activated, and catabolism-related genes are up-regulated to increase ATP synthesis. Meanwhile, cell growth and protein synthesis are inhibited by downregulating the mTOR pathway, and ATP consumption is reduced (7-9). In addition, AMPK activity is regulated by multiple upstream signals to coordinate its metabolism and specific energy requirements (10). Bone matrix formation and mineralization as well as bone absorption require a large amount of ATP (11,12), so activation of AMPK can effectively promote bone formation. In addition, AMPK has been reported to promote β-catenin transcription and autophagy through phosphorylation of histone deacetylase 5 (HDAC5) (13). The mTOR target protein is an evolutionarily highly conserved serine/threonine protein kinase which regulates a variety of cellular processes such as cell growth, cell cycle, cell survival, and autophagy. The mTOR consists of 2 functional complexes, among which mTORC1 exists as a multi-protein complex. The mTORC1 is directly regulated by cellular energy and nutrient status, and plays an important role in the regulation of autophagy (14).

Autophagy is a process of cell degradation and cycling in eukaryotes. Through the degradation of cytoplasmic organelles, protein molecules, and macromolecules, especially misfolded proteins and damaged organelles, products are recycled, which is of great significance for the survival of cells. In mammals, autophagy can be divided into microautophagy, megaphagy, and chaperone-mediated autophagy, and the degradation and recovery of the products of all 3 are carried out in lysosome (15). Expression of the ULK1 gene produces a serine/threonine protein kinase that interacts directly with FIP200 and is a key factor in autophagogenesis (16). Meanwhile, *Beclin1* is a homologous gene of yeast autophagy gene Atg6/Vps30 that mediates the localization of other phagoproteins to phagovesicles, thereby regulating the formation and maturation of mammalian autophagosomes (17,18). The *LC3* gene is autophagy-related, and LC3I can be modified to produce LC3II. This modification process is exacerbated by autophagy, so LC3II can be used as an indicator of autophagy (19). The Atg5 gene is an autophagy associated protein gene, which exists in most eukaryotes (20). At the early stage of autophagy formation, Atg5 protein binds to the outer membrane of autophagy to promote LC3 recruitment to autophagy (21). Mitochondrial autophagy is a kind of selective autophagy which is a common and normal physiological activity in healthy bodies, and can protect cells from apoptosis (22). Recently, the clinical study has shown that an abnormal level of autophagy can disrupt bone metabolism and play an important role in the occurrence of osteoporosis. The autophagy related genes are inhibited in osteoblasts, but activated in osteoclasts. In osteoporotic rats, autophagy regulation is unbalanced and abnormally activated, promoting the development of PMOP (23). Therefore, regulating the activity of autophagy and inhibiting its activation is a very important link in PMOP treatment. In this study, ZGZT was gavaged to PMOP rats to observe the effect of ZGZT on autophagy in PMOP rats, and to explore the therapeutic mechanism of ZGZT on postmenopausal osteoporosis in rats. We present the following article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-3724/rc).

Methods

Experimental animals and establishment of animal model

In this experiment, female rats were ovariectomized to construct PMOP animal model. Six-month-old SPF healthy female SD rats were provided by the Experimental Animal Center of Guangzhou University Medical College (Certificate No. 4400580009826). The animal experiment program was approved by the Animal Welfare and Ethical Management Committee of The Guangzhou University of Chinese Medicine (No. 20200317007), and was carried out according to the institutional guidelines for the care and use of experimental animals. All rats were freely adapted to feeding for 1 week in SPF Laboratory of Experimental Animal Center, College of Medicine, Guangzhou University (room temperature 25±1 ℃, relative humidity 50%±10%, light/dark cycle 12 hours). A protocol was prepared before the study without registration.

Model establishment and ZGZT administration

Female SD rats were randomly divided into 4 groups, with 12 rats in each group: control group, PMOP group, PMOP + ZGZT group and PMOP + E_2 group. Referring to previous literature (24), all PMOP rats received bilateral ovariectomy. To avoid wound infection, penicillin sodium (80,000 units/rat) was injected intramuscular to all rats undergoing surgery, once a day for 3 consecutive days. After 12 weeks, the normal control group and PMOP

group were given 10 mL/kg normal saline via intragastric administration every day. The ZGZT (Sichuan Meidakang Pharmaceutical Co., LTD, batch number: Z20050118) group was given ZGZT at a dose of 0.56 g/kg/d via intragastric administration. The E_2 group was given estrogen 0.104 mg/kg/d by gavage and 10 mL/kg normal saline was given via intragastric administration at other times.

Paw withdrawal threshold (PWT) determined

The von Frey filament method (North Coast Corporation, model: NC12775-99) was used to detect the PWT on 1 day before surgery, 1 month after surgery, 3 months after surgery, and 1 and 3 months after drug treatment (25). Before the test, the rats were acclimated for 1 h, and the medial and lateral plantar soles of both hind limbs were vertically stimulated from the lower part of the metal screen for 4–6 s. Foot retraction and foot licking were regarded as positive reactions, otherwise, they were regarded as negative reactions. The intensity of the stimulus started from 4 g, and when the intensity could not cause a positive reaction, the intensity of the stimulus was increased. If there was a positive reaction, the stimulus intensity is reduced, and the stimulus sequence was 0.4, 0.6, 1, 1.4, 2, 4, 6, 8, 10, 15, 26 g, until there was a positive and negative reaction bestriding. From the first positive reaction, continue to test 5 times according to the up and down method, the 50% PWT was obtained by literature algorithm (25). A total of 5 times of mechanical stimulation were measured. The interval of each mechanical stimulation was 30 s, and the maximum intensity was 15 g. If rats did not respond to a maximum of 15 g or a minimum of 0.4 g of filaments, 50% PWT was recorded as 15.0 g or 0.4 g, respectively.

Samples collection and treatment

After 12 weeks of administration, the rats were anesthetized by intraperitoneal injection of 3% chloral hydrate. We then collected 8 mL of blood from the abdominal aorta, centrifuged the supernatant at 3,000 rpm for 10 min, and stored it at –20 ℃ for future testing. After the rats were sacrificed, the lumbar vertebra tissues at L2–L5 level were taken and the surrounding soft tissues were removed. After rinsed with pre-cooled PBS, the tissues were placed in a 10 mL centrifuge tube, fixed with paraformaldehyde, and placed in the operating room –80 ℃ refrigerator for future testing. All operations were performed on ice, where specimens were stored at –20 ℃.

Bone mineral density was scanned by dual-energy X-ray absorptiometry

After modeling, dual energy X-ray absorptiometry (DXA; HLOGIC, USA) measuring bone mineral density (BMD). BMD of the lumbar spine (L1–L4) of PMOP rats was measured by DXA, and then the therapeutic effect of ZGZT on osteoporosis was evaluated.

Micro CT analysis of the third lumbar spine

The 3th lumbar was scanned by Micro CT (CT80, Scanco Medical, Switzerland) based on our scheme (26). In addition, the 3th lumbar tissue cross section were reconstructed using CTvox software (Bruker 3.2.0).

Histopathological examination of rat bone tissue by hematoxylin-eosin staining

The tissue sections of the 3th lumbar vertebra of dewaxed rat were fixed on slides and the section was dewaxed. The sections were then stained with hematoxylin (Beyotime, Shanghai, China) for 3 min, differentiated with 1% ethyl alcohol hydrochloride for 30 s, then stained with 0.5% eosin for 3 min. The sections were dehydrated with gradient ethanol and then xylene was added. Finally, after the sections were placed in the fume hood to dry, the sections were sealed with neutral gum. The histopathological morphological changes were observed under a microscope (Nikon, Tokyo, Japan).

Enzyme-linked immunosorbent assay detection of bone turnover markers, calcium, related hormones and cytokines, and inflammatory factors

The collected rat blood was stored at room temperature for 2 h, centrifuged at 1,000 rpm for 20 min, and the supernatant was taken. After that, serum tumor necrosis factor-α (TNF-α) concentration was detected with the enzyme-linked immunosorbent assay (ELISA) kit (Thermo, MULTISKAN MK3, USA), and the detection procedure was carried out according to the instructions. Interleukin 17 (IL-17), IL-1β, bone alkaline phosphatase (B-ALP), Procollagen 1 intact N-terminal propeptide (PINP), cross-linked C-telopeptide of type 1 collagen (CTX) and blood calcium were detected by the corresponding kit, and the operation was the same as above.

Tartrate-resistant acid phosphatase staining to detect osteoclast activity

The slices were successively soaked in xylene for 20 min, anhydrous ethanol for 20 min, anhydrous ethanol for 5 min, 75% alcohol for 5 min, and then cleaned with tap water and distilled water 3 times. We then mixed 500 μL of sodium nitrate and vice fuchsin solution evenly, added 18 mL of sodium acetate buffer solution and mixed well, then added 1 mL of naphthol AS-BI phosphate ester solution, weighed and added 0.28 g of sodium potassium tartrate, and ensured that the working solution was fully dissolved. Dripping working solution was added to the tissue sections, incubated at 37 ℃ for 1–2 h. The slices were dyed with hematoxylin dyeing solution for 3–5 min, differentiated with differentiation solution, returned blue with returned blue solution. The slices were soaked in anhydrous ethanol for 5 min and repeated for 3 times. Then, the tablets were put into xylene for 5 min and sealed with neutral gum. The slices were then observed under the microscope and images were acquired.

Western blot detection of the expression levels of mTOR, AMPK, ATG-5, Beclin 1, LC3 II, and ULK1 in 5th lumbar bone tissue of rats

The 5th lumbar bone tissue of rats was taken and placed in a tissue homogenizer, with the addition of precooled radioimmunoprecipitation assay (RIPA) lysate and protease inhibitor (Ford Biology, Hangzhou, China), tissue homogenate, and centrifuged at 4 ℃ at 12,000 rpm for 20 min. The protein concentration was determined by bicinchoninic acid (BCA) method, and the sample with the measured concentration was used to calculate the volume with the mass first. Due to the different volume of each sample, RIPA was added to the equal volume, so that the volume and concentration were the same, and then an equal volume of sodium dodecyl sulfate (SDS) was added. The protein samples were mixed with 2× SDS buffer (Boster, Wuhan, China) in equal volume, and the protein was denatured at 95 ℃ for 5 min. Then, the protein was stored in an ice bath for 10 min at –20 ℃ for reserve. Proteins were isolated by SDS-polyacrylamide gel electrophoresis (PAGE). The 20 μg protein sample was transferred to nitrate cellulose film (Millipore, Burlington, MA, USA) at 100 V for 1 h by 10% SDS-PAGE, and then sealed at 37 ℃ for 1 h in sealing solution, followed by incubation with the primary antibody (1:1,000/1:2,000, Beyotime) at 4 ℃ overnight. After washing with tris-buffered saline with Tween 20 (TBST) for 3× 5 min, the secondary antibody (1:1,000/1:2,000, Beyotime) was added for 1 h at room temperature, followed by washing in TBST again for 3× 5 min. Western blotting (WB) and image analysis were used to determine the absorbance (A) of each band for quantitative analysis.

Detection of the expression levels of mTOR, AMPK, ATG-5, Beclin 1, LC3 II, and ULK1 in 5th lumbar bone tissue by quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

The total RNA was extracted with Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA). Then, 5 μL of total RNA was extracted, and complementary DNA (cDNA) was synthesized in 20 μL reverse transcription system. Primers of upstream and downstream target genes were added with 5 μL cDNA as a template. According to the instructions of the TaKaRa qRT-PCR reaction kit (qRT-PCR; Takara, Shiga, Japan), the PCR reaction solution was prepared, and the PCR dissolution curve was detected and relatively quantified. Glyceraldehyde 3-phospahte dehydrogenase (GAPDH) was used as the internal reference. The results were analyzed using ABI 7500 Real-Time PCR System (Thermo Fisher, USA).

Statistical analysis

The results were expressed as mean ± SD (standard deviation), analyzed by SPSS20.0 (SPSS lnc., IBM, USA), and were drawn by GraphPad Prism software 8.2.1 (GraphPad Software, Inc., La Jolla, CA, USA). The Image J software processing system (National Institutes of Health, Bethesda, MD, USA) was used to analyze the bands and optical density of WB results. All data were tested for normality and homogeneity of variance. If the variance was equal, Tukey (W) method was used if the variance was not equal, the Games-Howell (A) method was used for pairwise comparison among groups. $P<0.05$ was considered statistically significant.

Results

ZGZT Capsule can improve the mechanical pain threshold of PMOP rats

The mechanical pain threshold of PMOP rats was observed

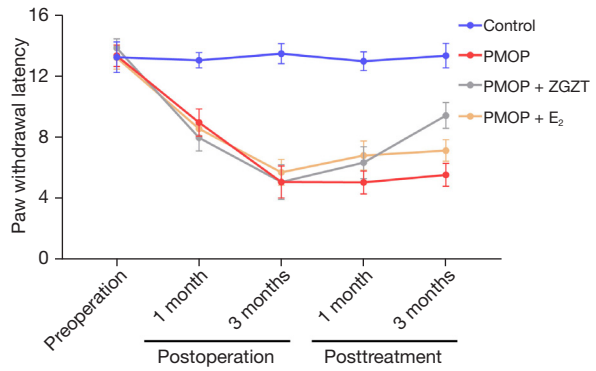

Figure 1 Effect of ZGZT Capsule on mechanical pain threshold in PMOP rats. There was no significant change in the mechanical pain threshold in the control group, but the mechanical pain threshold in the PMOP group, the PMOP + ZGZT group and the PMOP + E_2 group decreased significantly at 1 and 3 months after operation. Compared with PMOP group, PMOP + ZGZT group and PMOP + E_2 group could partially alleviate the reduction of pain threshold after 1 and 3 months of treatment, and PMOP + ZGZT group had the most significant in alleviate the reduction of pain threshold after 3 months of treatment. PMOP, Postmenopausal osteoporosis; ZGZT, Zhuanggu Zhitong; E_2, estrogen.

by Von Frey filaments method. As shown in *Figure 1*, the results showed that compared with the control group, the mechanical pain threshold of PMOP rats was significantly lower at the 1st and 3rd month after surgery (the last day of each month). Compared with the PMOP group, the mechanical pain threshold of PMOP + ZGZT group was partially alleviated at the 1st and 3rd month after treatment.

ZGZT can effectively improve osteoporosis in PMOP rats

Decreased BMD, decreased trabecular number, and decreased bone thickness are significant features of osteoporosis. The BMD measurements of rats showed a slight decrease in the PMOP group compared with the normal group (*Figure 2A*). The bone mass of the PMOP + ZGZT group and PMOP + E_2 group had no significant change compared with the control group. The CT scan showed that the third lumbar vertebra bone tissue in the PMOP group was significantly deformed (*Figure 2B*). Hematoxylin and eosin (HE) staining results showed that fat vacuoles were significantly increased in the PMOP group, but no significant changes were observed in the PMOP + ZGZT and the PMOP + E_2 group (*Figure 2C*).

ZGZT can improve bone trabecular density of rat lumbar vertebrae

Compared with the control group, bone trabecular thinning and fracture were observed in the PMOP group, indicating that osteoporosis was obvious in the PMOP group. The bone condition of PMOP + ZGZT group and PMOP + E_2 group was similar. And the trabecular bone of PMOP + ZGZT group and PMOP + E_2 group was denser than that of PMOP group, but the trabecular bone was slightly loose compared with that of control group. Compared with PMOP + E_2 group, the bone trabecular arrangement of PMOP + ZGZT group was more regular and orderly, indicating that both PMOP + ZGZT group and PMOP + E_2 group could partially alleviate osteoporosis (*Figure 3*).

ZGZT can restore bone metabolism

Low levels of calcium in the serum lead to insufficient calcium absorption and elevated B-ALP levels. The CTX levels reflect bone resorption levels and are significantly elevated in patients with osteoporosis. The PINP levels reflect the rate of type I collagen synthesis and osteogenic activity, and are elevated in patients with osteoporosis. Serum levels of calcium, B-ALP, PINP, and CTX were determined by ELISA. The results showed that the serum calcium content in the PMOP group was significantly decreased, the levels of B-ALP and PINP were significantly lower than those in the control group, while CTX level had no significant change. There were no significant differences in the levels of B-ALP, PINP, CTX in the PMOP + ZGZT group and PMOP + E_2 group compared with those in the control group (*Figure 4A-4D*). The level of tartrate-resistant acid phosphatase (TRAP) was determined by TRAP staining, and the results showed that the TRAP level in the PMOP group was significantly higher than that in the control group (*Figure 4E*). This indicates that in the PMOP group, the bone formation level was decreased, while the bone absorption level was increased, and ZGZT can effectively restore bone metabolism.

ZGZT can effectively reduce the release of inflammatory factors

The inflammatory cytokines TNF-α, IL-17, and IL-1β can lead to osteoclast activation, which leads to local osteolysis. Moreover, inflammatory factors are closely related to the pain caused by osteoporosis, and the level of inflammatory

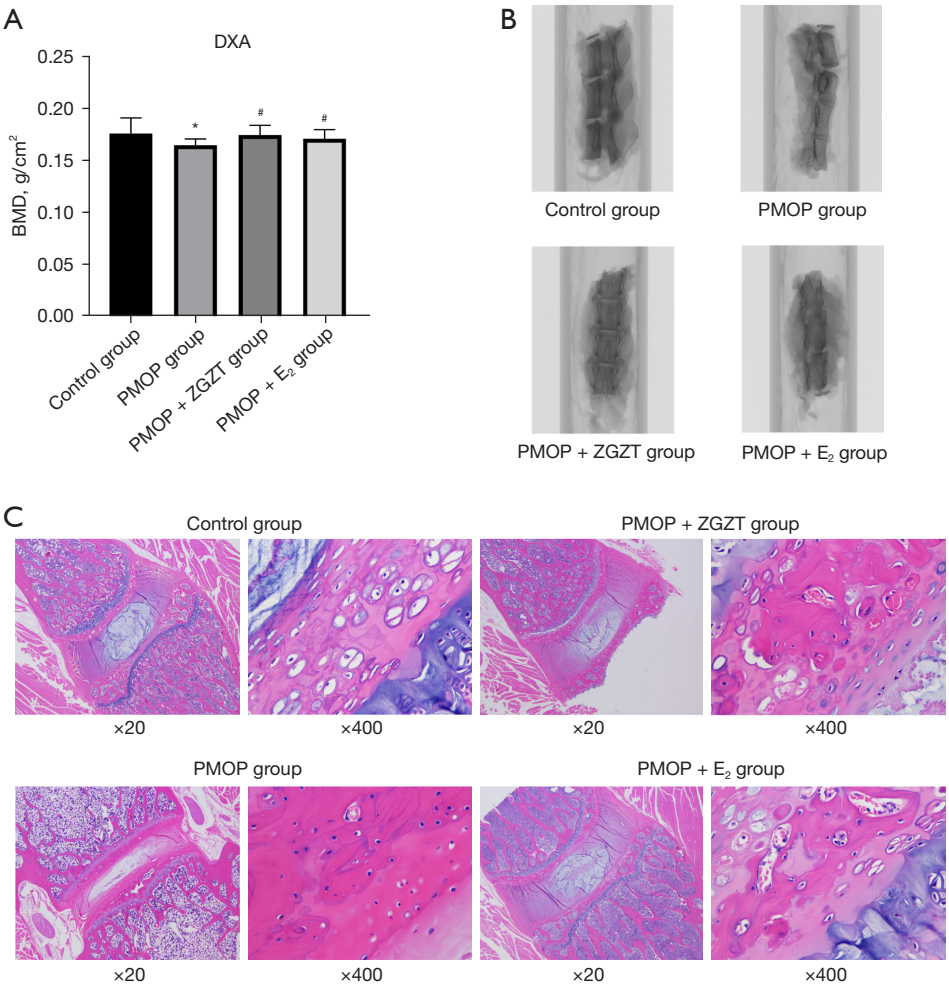

Figure 2 Effect of ZGZT Capsule on BMD, femur morphology and fat vacuole in rats with osteoporosis. (A) Average BMD of rats in each group; (B) CT results, the femurs of rats in the PMOP group were significantly deformed; (C) HE staining results showed that fat vacuoles were significantly increased in the PMOP group. *P<0.05 compared with the control group; #P<0.05 compared with the PMOP group. BMD, bone mineral density; CT, computed tomography; HE, hematoxylin and eosin; PMOP, postmenopausal osteoporosis; ZGZT, Zhuanggu Zhitong; E_2, estrogen; DXA, dual energy X-ray absorptiometry.

factors can indirectly indicate the intensity of pain, thus their measurement may verify the analgesic effect of ZGZT. Serum levels of TNF-α, IL-17, and IL-1β were detected by ELISA. The expression levels of TNF-α and IL-1β in the PMOP group were higher than those in the Control group, while the expression levels of IL-17 in the PMOP group were lower than those in the Control group. The expression levels of TNF-α, IL-1β, and IL-17 in the PMOP + E_2 group and the PMOP + ZGZT group were lower than those in the control group (*Figure 5A-5C*). The results showed that ZGZT could effectively inhibit the release of inflammatory factors and relieve pain.

ZGZT can effectively increase bone tissue cell autophagy

All of Atg-5, Beclin 1, LC3 II, and ULK1 are autophagy related factors. The mTOR can inhibit autophagy, AMPK can activate autophagy, and they jointly regulate autophagy. Increased autophagy of osteoblasts can reduce apoptosis and improve survival time. The expression levels of mTOR, AMPK, ATG-5, Beclin 1, LC3 II, and ULK1 in the L5 lumbar bone tissue of rats were detected by WB and qRT-PCR (*Figure 6A,6B*). In the PMOP group, mTOR expression level was high, AMPK level was low, and the related autophagy markers ATG-5, Beclin 1, LC3 II, and

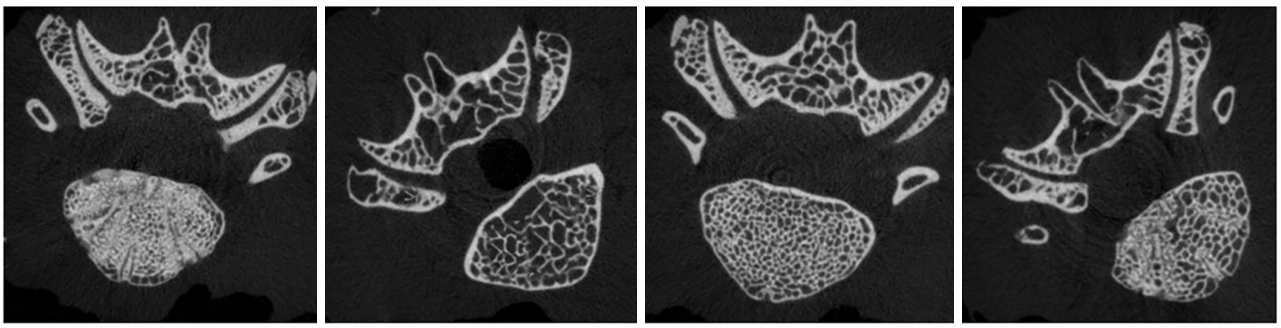

Figure 3 Micro CT cross-sectional scanning image of lumbar vertebrae in rats. Compared with the control group, the osteoporosis was obvious in the PMOP group. And the trabecular bone of PMOP + ZGZT group and PMOP + E_2 group was denser than that of PMOP group, but the trabecular bone was slightly loose compared with that of control group. Both PMOP + ZGZT group and PMOP + E_2 group could partially alleviate osteoporosis. PMOP, postmenopausal osteoporosis; ZGZT, Zhuanggu Zhitong; E_2, estrogen.

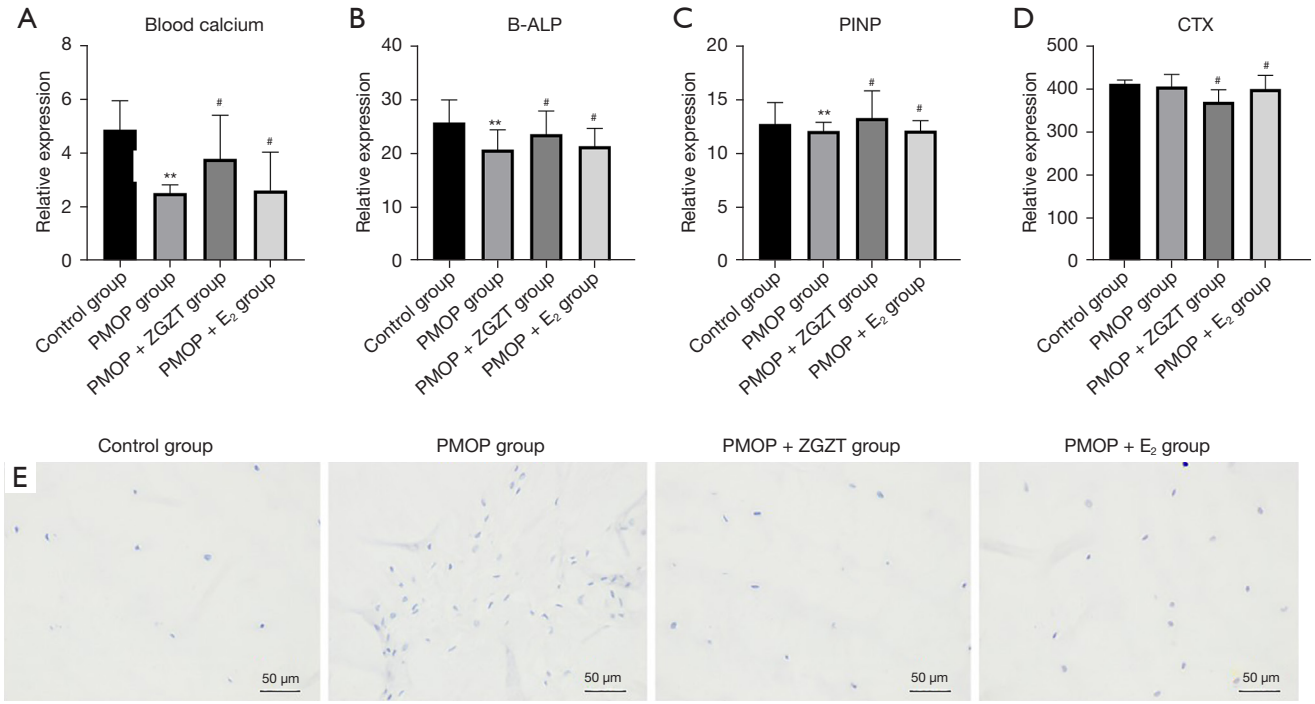

Figure 4 Effects of ZGZT on blood calcium, B-ALP, PINP and CTX levels in rats. (A) Compared with the control group, blood calcium in the PMOP group was significantly reduced. After intervention, the serum calcium content the PMOP + ZGZT and PMOP + E_2 group increased significantly compared with the PMOP group. (B,C) PINP and B-ALP showed the same trend. The expression levels of PINP and B-ALP in the PMOP group were decreased compared with that in the control group, and the expression levels of PINP and B-ALP in the PMOP + ZGZT and PMOP + E_2 group were up-regulated after intervention, but there was no statistical significance. (D) There was no significant change in CTX in the PMOP group, and the expression of CTX in the PMOP + ZGZT group was decreased. (E) The number of TRAP staining positive cells in the PMOP group was significantly increased, the number of positive cells in the PMOP + ZGZT group was not significantly different from that in the control group, and the number of positive cells in the PMOP + E_2 group was increased compared with that in the control group, but significantly lower than that in the PMOP group. **P<0.01, compared with the control group; #P<0.05, compared with the PMOP group. PINP, procollagen 1 intact N-terminal propeptide; B-ALP, bone alkaline phosphatase; CTX, C-telopeptide of type 1 collagen; PMOP, postmenopausal osteoporosis; ZGZT, Zhuanggu Zhitong; TRAP, tartrate-resistant acid phosphatase; E_2, estrogen.

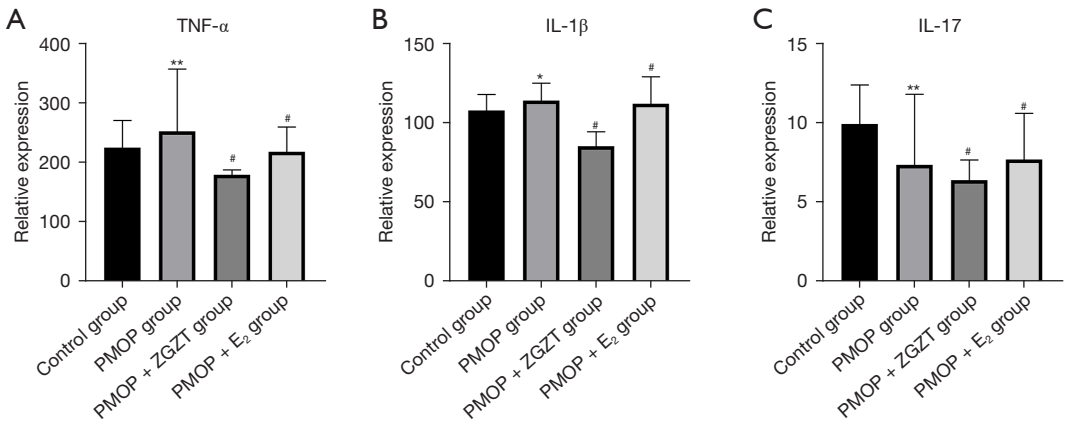

Figure 5 Effect of ZGZT on serum levels of inflammatory factors TNF-α, IL-1β and IL-17. (A) The expression level of TNF-α increased in the PMOP group, decreased in the PMOP + ZGZT group, and showed no significant change in the PMOP + E_2 group; (B) compared with the control group, the expression level of IL-1β in the PMOP group increased, while that in the PMOP + ZGZT group decreased, and there was no significant change in the PMOP + E_2 group; (C) the expression of IL-17 in both the PMOP group and the PMOP + E_2 group was decreased, and the expression level was similar, and the expression level of PMOP + ZGZT group was lower than that of the PMOP group. *P<0.05, **P<0.01, compared with control group; #P<0.05, compared with the PMOP group. TNF-α, tumor necrosis factor-α; IL-1β, interleukin 1β; IL-17, interleukin 17; PMOP, postmenopausal osteoporosis; ZGZT, Zhuanggu Zhitong; E_2, estrogen.

ULK1 expression levels were low. In the PMOP + ZGZT group and PMOP + E_2 group, the mTOR level was lower, and the expression level of AMPK and related autophagy markers ATG-5, Beclin 1, LC3 II, and ULK1 was higher than in the PMOP group. Therefore, ZGZT can effectively activate the AMPK pathway, inhibit the mTOR pathway, and improve autophagy.

Discussion

In this study, it was reported for the first time that ZGZT could effectively improve BMD in PMOP rats and improve the mechanical withdrawal threshold of PMOP rats to play an analgesic role. The mechanism may be that ZGZT can promote AMPK expression, inhibit mTOR expression, regulate bone metabolism balance and inhibit the release of inflammatory factors, thus playing the role of "bone strengthening" and "pain relieving".

So far, a number of studies have proved that autophagy is involved in the formation and differentiation of osteoblasts and osteoclasts in the process of bone reconstruction, and plays an important role in the pathogenesis of OP (26,27). AMPK/mTOR signaling pathway is one of the bridges linking intracellular and intracellular signaling and cellular response effect, is widely exists in various cells of the body, and is involved in cell metabolism, proliferation, differentiation, autophagy, apoptosis and other physiological and pathological process. AMPK/mTOR signaling pathway is also a classical signaling pathway of autophagy, which not only indirectly regulates the functions of osteoblasts and osteoclasts, but also directly participates in the formation of osteoblast mineralization and osteoclast frill (28). A study has shown that activation of AKT/mTOR signaling pathway can inhibit autophagy, promote cell differentiation, and ultimately play an anti-osteoporosis role (29). AMPK is also a recognized autophagy regulator that promotes autophagy by inhibiting phosphorylation activation of its downstream target of rapamycin receptors (30). mTOR is an evolutionarily highly conserved serine/threonine protein kinase that regulates a variety of cellular processes and can be directly regulated by cell energy and nutritional status, and plays an important role in the regulation of autophagy (31). Therefore, activation of AMPK/mTOR signaling pathway can play a good therapeutic effect on PMOP, and both AMPK and mTOR can be used as therapeutic targets of PMOP.

ULK-1 is another key regulatory protein of autophagy that is regulated by upstream AMPK and mammalian target of rapamycin (mTOR) (32). When the body lacks energy, such as hypoxia, AMPK can directly activate ULK-1 to improve autophagy and promote cell survival. mTOR negatively regulates ULK-1, when p-mTOR level increases, p-mTOR can block the interaction between ULK-1 and AMPK, thus inhibiting autophagy.

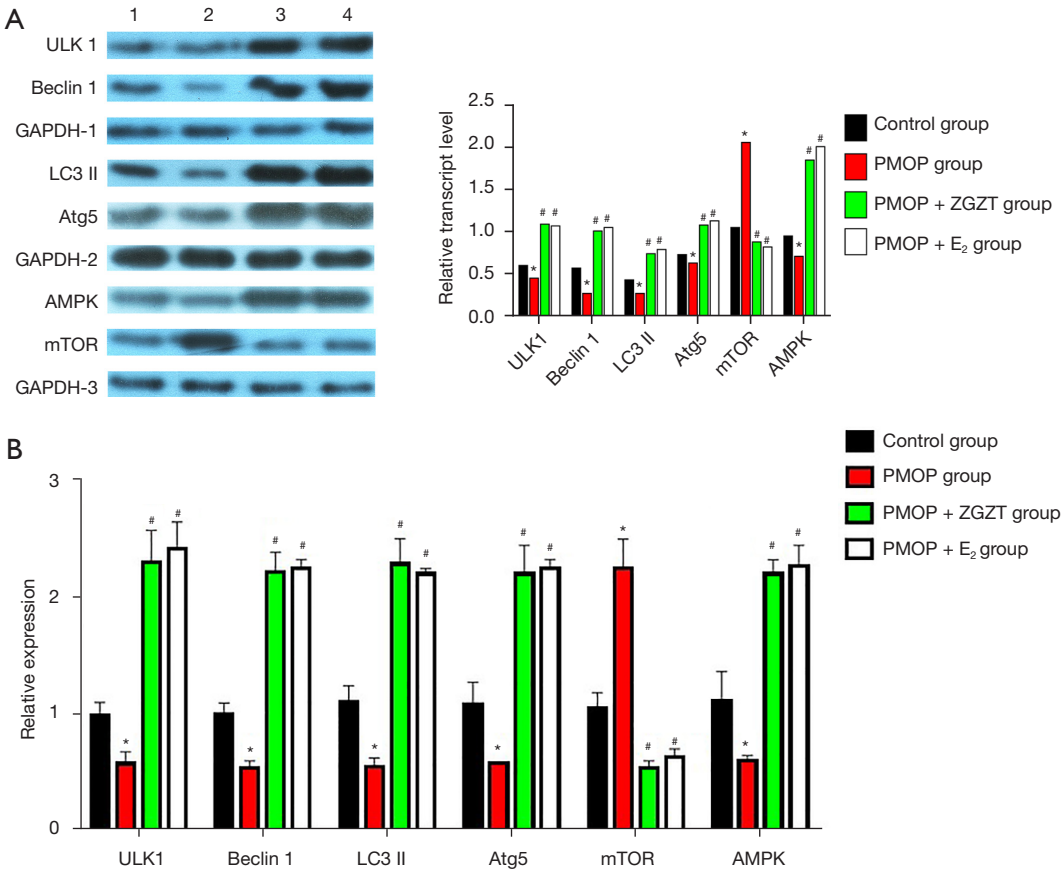

Figure 6 Effect of ZGZT on autophagy-associated proteins. (A) Autophagy-related factors and the protein expression levels of AMPK and mTOR (the internal reference gene of ULK 1 and Beclin 1 was GAPDH-1. The reference gene of LC3 II and Atg5 was GAPDH-2. The internal reference gene of AMPK and mTOR was GAPDH-3) (1: Control group; 2: PMOP group; 3: PMOP + ZGZT group; 4: PMOP + E_2 group). (B) Autophagy-related factors and the relative mRNA expression levels of AMPK and mTOR. *P<0.05 compared with control group; #P<0.05 compared with the PMOP group. PMOP, Postmenopausal osteoporosis; ZGZT, Zhuanggu Zhitong; E_2, estrogen.

In addition, autophagy can inhibit the proliferation and differentiation of osteoclasts by regulating T cells and inhibiting the release of inflammatory factors, among which inflammatory factors and osteoclasts play a very important role in osteoporotic pain (33,34). Xu *et al.* found that ozone can regulate the autophagy of OA chondrocytes through AMPK/mTOR signaling pathway, thereby inhibiting the release of inflammatory factors, balancing chondrocyte synthesis and catabolism, delaying the degeneration of articular cartilage, and activating AMPK in lumbar bone tissue to play an analgesic role, it suggesting that inflammatory factors are the main factors causing pain in articular cartilage (35). AMPK/mTOR signaling pathway mediated autophagy plays a major role in analgesia (36,37). This part of the experiment verified that mechanical withdrawal threshold was significantly reduced in PMOP rats, and ZGZT could partially relieve mechanical pain caused by osteoporosis, while AMPK/mTOR autophagy signaling pathway was significantly activated after ZGZT intervention, so pain behavior may also be one of the activated phenotypes of AMPK/mTOR autophagy signaling pathway.

ZGZT, a kind of Proprietary Chinese Medicine, has been reported to successfully increase BMD and relieve osteoporotic pain in many OP patients (3,38). According to literature reports, the reasonable dose of ZGZT Capsule for rats is 0.5625 g/kg, so the dose used in this study is 0.56 g/kg, which is relatively safe (2). In the early stage, the research group predicted the molecular mechanism of ZGZT treatment of PMOP through network pharmacology, including mTOR signaling pathway, TNF signaling pathway and IL-17 signaling pathway. Animal

experimental results showed that ZGZT can effectively relieve pain and improve bone mineral density and bone microstructure in PMOP rats, suggesting that ZGZT may be one of the potential drugs to treat PMOP in the future, although a large number of high-quality RCT studies are still needed to confirm these results.

The current study has some limitations. First, we used only female SD rats from the same source for the study. At present, there is no suitable rat model of postmenopausal PMOP with pain in addition to natural aging. How pain and osteoporosis form feedback pathways through autophagy and regulate each other needs further study. Secondly, due to the lack of an appropriate PMOP cell model, we did not use pathway inhibitors to further verify the molecular mechanism of pathway and ZGZT therapy, which requires further research in the future.

Conclusions

We successfully constructed a PMOP rat model and found that the molecular mechanism of pathogenesis and treatment of osteoporosis and pain in PMOP rats is closely related to AMPK/mTOR signaling pathway. ZGZT can significantly reduce PMOP by up-regulating AMPK/mTOR signaling pathway, reduce the release of inflammatory factors and increase mechanical withdrawal threshold to play an analgesic role. In theory and practice, this study provides a new therapeutic option for ZGZT to prevent and treat PMOP.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-3724/rc

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-3724/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The animal experiment program was approved by the Animal Welfare and Ethical Management Committee of The Guangzhou University of Chinese Medicine (No. 20200317007), and was carried out according to the institutional guidelines for the care and use of experimental animals.

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