Tougu Xiaotong capsule exerts a therapeutic effect on knee osteoarthritis by regulating subchondral bone remodeling

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Received November 12, 2018; Accepted December 12, 2018

DOI: 10.3892/mmr.2018.9778

Abstract. Previous studies have shown that Tougu Xiaotong capsule (TGXTC) has therapeutic effects on knee osteoarthritis (OA) through multiple targets. However, the mechanisms of action underlying its regulation of subchondral bone reconstruction remain unclear. In this study, we investigated the effects of TGXTC on subchondral bone remodeling. Eighteen six-month-old New Zealand white rabbits of average sex were randomly divided into the normal, model and TGXTC groups. The rabbit knee OA model was induced by a modified Hulth's method in the model and TGXTC groups, but not the normal group. Five weeks postoperatively, intragastric administration of TGXTC was performed for four weeks. After drug administration, the medial femoral condyle and tibia were prepared for observation of cartilage histology via optical microscopy and micro-computed tomography, the serum was collected for biochemical parameters assay and the subchondral bone isolated from the lateral femoral condyle was collected for detection of IL-1β and TNF-α mRNA and protein by reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. The results showed that treatment with TGXTC significantly mitigated cartilage injury and subchondral bone damage, improved the parameter of subchondral trabecular bone, decreased alkaline phosphatase and tartrate-resistant acid phosphatase activity, and significantly reducing the osteoprotegerin/receptor activator of nuclear factor-κB ligand ratio, reduced the expression of IL-1β and TNF-α mRNA and protein. These results suggest that TGXTC could delay the pathological development of OA by regulating subchondral bone remodeling through regulation of bone formation and bone resorption and its relating inflammatory factors, and this may partly explain its clinical efficacy in the treatment of knee OA.

Introduction

Osteoarthritis (OA) is characterized by the degradation and loss of articular cartilage, inflammation of the synovial membrane, osteophyte formation and changes in the subchondral bone (1-4). Previous studies focused on cartilage, the subchondral bone was recently found to play a crucial role in OA (5,6). Subchondral bone loss occurs during the early stage of OA while subchondral sclerosis occurs in the late stage of OA, along with the presence of osteophytes (7). In addition, a vicious cycle between structural changes in the subchondral bone and cartilage damage occurs, which contributes to the progression of OA. In a word, the subchondral bone is another interesting intervention point for the treatment of OA.

Therefore, to understand the microstructure of the subchondral bone is very important in OA. Micro-computed tomography (micro-CT) can detect microstructural changes and measure parameters of subchondral bone (8). The most important change in the subchondral bone is the trabecular bone. Therefore, it is necessary to evaluate the structural changes of trabecular bone and its related parameters by micro-CT. Moreover, the parameters and structural changes of subchondral bone are related to bone remodeling (9,10).

Bone remodeling plays an essential role in the balance of formation and resorption in bone tissue (11). In this regard, alkaline phosphatase (ALP) can enhance osteoblast differentiation and promote bone formation (12,13). Tartrate-resistant acid phosphatase (TRAP) is used as a marker for osteoclasts and bone resorption (14). The osteoprotegerin (OPG)/receptor activator of nuclear factor-κB ligand (RANKL) system is one of the most essential molecular mechanisms to regulate bone remodeling. Imbalances in the OPG/
RANKL system can cause abnormal bone metabolism and subchondral bone damage (15-17). The OPG/RANKL ratio is vital to the bone mass because it maintains normal bone turnover (18,19). Previous findings have shown that regulation of bone metabolism occurs through the classical OPG/RANK/RANKL pathway, the direct and indirect functions of which are influenced by numerous factors, including IL-1β and TNF-α (20,21).

Tougu Xiaotong capsule (TGXTC) is an effective prescription in OA treatment which consists of Ligusticum chuanxiong, Morinda officinalis, Sarcedra glabra and Paeonia lactiflora (22,23). There are many effective molecules in TGXTC, which is multi-drug, multi-path and multi-target of molecular mechanism for the treatment of OA, and the non-linear regulation pattern exists in TGXTC ligand-target interaction network (24). Previous studies have proved that TGXTC has therapeutic effects on knee OA (25) through multiple targets, such as inhibiting chondrocyte apoptosis (26), improving the structure and function of cartilage (27) and slowing down cartilage degradation (23), promoting osteoblast proliferation and calcium secretion (22). However, evaluation of the effect of TGXTC on subchondral bone remodeling using micro-CT combined with markers of bone formation and bone resorption has not been reported.

To further elucidate the precise mechanism of the potential treatment of OA, in the present study, we investigated the effects of TGXTC in the bone remodeling of subchondral bone on the basis of micro-CT assessment of trabecular bone. We found could TGXTC delay the pathological development of OA through regulating formation/resorption balance and it's relating inflammatory factor to improve the remodeling of subchondral bone.

Materials and methods

Animals. A total of 18 female New Zealand rabbits (age, 6 months; weight, 1.7-2.3 kg) were purchased from the Shanghai City Songjiang District Songlian Experimental Animal Farm (Shanghai, China). These animals were bred in the Animal Center of Fujian University of Traditional Chinese Medicine (Fujian, China). The present study was approved by the Animal Care and Use Committee of the Fujian University of Traditional Chinese Medicine (Fujian, China).

Drugs and reagents. TGXTC was prepared by the Second People's Hospital of Fujian University of Traditional Chinese Medicine (Fujian, China) (approval no. MIN ZIZHI Z201000006). Sodium pentobarbital was obtained from Shanghai Xitang Biotechnology Co., Ltd. (Shanghai, China), sodium penicillin was purchased from GE Healthcare Life Sciences (Logan, UT, USA), paraformaldehyde was from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China), and EDTA was provided by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The ALP and tartrate resistant acid phosphatase assay kits were obtained from Beyotime Institute of Biotechnology (Nantong, China). The rabbit OPG ELISA kit and rabbit RANKL ELISA kit were purchased from Shanghai Westang Bio-Tech Co., Ltd. (Shanghai, China). Other reagents included TRIzol, which was obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). PrimeScript™ RT reagent kit with gDNA Eraser and SYBR® Premix Ex Taq TM II were purchased from Takara Bio., Inc. (Osaka, Japan), mouse anti-β-actin antibody (monoclonal; 1:8,000; cat. no. HC201), goat anti-mouse horseradish peroxidase-conjugated IgG (monoclonal; 1:4,000; cat. no. HS201) and goat anti-rabbit horseradish peroxidase-conjugated IgG (monoclonal, 1:4,000; cat. no. HS101) were purchased from TransGen Biotech Co., Ltd. (Beijing, China), rabbit anti-IL-1β antibody (polyclonal; 1:500; cat. no. 16806-1-AP) was provided by Wuhan Sanying Biotechnology (Hubei, China), and rabbit anti-TNF-α (monoclonal; 1:500; cat. no. MAB2103) antibody was obtained from R&D Systems China (Shanghai, China). All other chemicals, unless otherwise stated, were obtained from Sigma-Aldrich (Merck KGaA, St. Louis, MO, USA).

Grouping, model preparation and treatments. The 18 rabbits were randomly divided into three groups, including the normal, model and TGXTC group, with 6 rabbits in each group.

The rabbit OA model was established by using modified Hulth's method in all groups except for the normal group (28). Rabbits were anesthetized by ear vein injection with sodium pentobarbital (30 mg/kg). After a routine disinfection, the medial collateral and anterior cruciate ligaments were transected via the medial approach, and the medial meniscus was excised. Then the joint capsule was sutured layer by layer. Prophylactic antibiotic with sodium penicillin (400,000 units) was given for three days after surgery. After a week, the animals were forced to movement for 30 min daily for one month.

After five weeks, a clinical oral dose of TGXTC (140 mg/kg/day) was given to rabbits in the TGXTC group for four consecutive weeks. An equivalent volume of saline was administered to the normal and model group rabbits.

Specimen collection. After intragastric drug administration, the rabbits were sacrificed by air embolism after anesthesia with 3% sodium pentobarbital (30 mg/kg ear marginal vein injection). The femur, tibia and serum were harvested. The medial femoral condyle was prepared for Safranin O-fast green staining and the tibia for micro-CT, respectively. The serum was collected for biochemical parameters assay. The subchondral bone isolated from the lateral femoral condyle was collected for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis.

Histology. The medial femoral condyle tissues were fixed in 4% paraformaldehyde for 3 days and decalcified in 10% EDTA at room temperature for about 4 months. The medial femoral condyle was longitudinally cut and embedded in paraffin. Sagittal sections (4 μm) were prepared for Safranin O-fast green staining and observed under a light microscope (DM4000 B; Leica Microsystems GmbH, Wetzlar, Germany) and images were captured at a magnification, x100. Following that a modified Mankin scoring principles (29) was used to evaluate the degeneration of each femoral condyle.

Micro-CT evaluation. A micro-CT system (ZKKS-MCT; Zhongke Kaisheng Medical Technology Co., Ltd., Guangzhou, China), with a source voltage of 60 kV, a power of 40 W, a current of 666 μA, and resolution of 50 μm, was used to obtain
X-ray radiographs. The specimens were attached to a stage that rotated 360° with images captured every 0.72°. A 3D analysis was used to calculate parameters of the tibial in the region at 4.2x1.2x1.8 mm from the subchondral bone. The parameters measured were: Tabular number (Tb.N), trabecular thickness (Tb.Th), bone volume fraction (BV/TV) and trabecular separation (Tb.Sp).

Serum biochemistry assay. After treatment, blood samples were collected and centrifuged at 3,000 x g for 10 min at 4°C. The supernatants were stored at -20°C until analysis. ALP and TRAP activity was measured with autobiochemical analyzer by standard colorimetric methods using detection kits, according to the manufacturer’s protocol. Serum OPG and RANKL levels were observed by ELISA kit, according to the manufacturer's protocol.

RT-qPCR. Total RNA was extracted from the subchondral bone of the lateral femoral condyle using TRIzol and quantified using a BioPhotometer Plus UV spectrophotometer (Eppendorf AG, Hamburg, Germany). cDNA was synthesized using a Takara PrimeScript™ RT reagent kit with gDNA Eraser. Primers were designed and produced by Takara Bio., Inc. (Table I). The PCR system was prepared according to the manufacturer’s instructions, with 10 µl of SYBR® Premix Ex Taq II, 0.4 µl of ROX Reference Dye II, 0.8 µl of upstream primer, 0.8 µl of downstream primer, 2 µl of cDNA, and 6 µl of dH2O, 20 µl in total. The PCR amplification protocol was: Pre-denaturation at 95°C for 30 sec, denaturation at 95°C for 3 sec, and annealing at 60°C for 30 sec, for a total of 40 cycles. The fluorescence signal of GAPDH acted as an internal reference for calculating the relative gene expression levels.

Western blot analysis. The subchondral bone isolated from the lateral femoral condyle was immersed 1:10 in lysis buffer containing 50mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.1% sodium orthovanadate and 2 mM EDTA (Beijing BLKW Biotechnology Co., Ltd., Shanghai, China), homogenized using a Tissuelyser-192 (Shanghai Jingxin Industrial Development Co., Ltd., Shanghai, China) on ice, then centrifuged at 4°C at 12,000 x g for 30 min. A bicinchoninic acid kit (cat. no. P0010; Beyotime Institute of Biotechnology) was applied to determine protein concentrations. Protein samples (30 µg) were electro-phoresed at 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis for 2 h (Beyotime Institute of Biotechnology, Shanghai, China), transferred onto polyvinylidene difluoride (Shanghai Jinghong Laboratory Instrument Co., Ltd.) membranes, and blocked with 5% skimmed milk for 2 h. Next, the samples were incubated on a shaker at 4°C overnight with primary antibodies against IL-1β (1:500), TNF-α (1:500) and β-actin (1:8,000). The samples were then rinsed with TBST, incubated with their corresponding secondary antibodies: Goat anti-mouse horseradish peroxidase-conjugated IgG (monoclonal; 1:4,000; cat. no. HS201) or goat anti-rabbit horseradish peroxidase-conjugated IgG (monoclonal; 1:4,000; cat. no. HS101) both from TransGen Biotech, Inc., on a shaker at room temperature for 1 h, rinsed with TBST, and developed using ECL substrate. Image processing was carried out using the ChemiDoc™ XRS + system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with BeyoECL Plus (Beyotime Institute of Biotechnology) to analyze gray values and to determine the relative expression of the proteins.

Statistical analysis. Data were expressed as mean ± standard deviation and were analyzed by SPSS version 20.0 software. The Shapiro-Wilk test was used to determine the normality of all groups of data. If the data exhibited a normal distribution, they were analyzed with one-way analysis of variance (ANOVA) followed by least significant difference or Games Howell post hoc tests. If not, the Kruskal-Wallis test was used and the Mann-Whitney U with Bonferroni's correction was applied as a post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

TGXT decreased cartilage damage. To determine the effects of TGXT on cartilage morphology, the sections were stained with Safranin-O-fast green and observed under an optical microscope. As shown in Fig. 1A, the superficial zone, mid zone, deep zone, and calcified cartilage were visible; the surface of articular cartilage was smooth and had no cracks, the chondrocytes were arranged in rows, and the tidemark and cement line were clear. The proteoglycans were stained by Safranin O, and fast green staining was evident in the superficial zone and the subchondral bone in the normal group. In the model group, the cartilage surface was damaged with cracks and disordered chondrocyte clusters, and the tidemark replication. In addition, staining of proteoglycans was severely reduced (Fig. 1B). However, following treatment with TGXT, the articular cartilage had little rough surface without cracks, chondrocytes arrangement were arranged in order, and the cartilage matrix stained became deeper and even better than the normal group (Fig. 1C).

We used the scoring principles of Mankin to analyze the change of cartilage degeneration. The Mankin score in the model group was significantly higher compared with the normal group (P<0.01). However, the Mankin score was

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<th>Genes</th>
<th>Sequence</th>
<th>Product length (bp)</th>
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<tr>
<td>IL-1β</td>
<td>F: 5'-AACAAGTGGTGT&lt;br&gt;TCTCCATGAGTTT-3'&lt;br&gt;R: 5'-GGGTAGGCTTA&lt;br&gt;TGCCTCATCAC-3'</td>
<td>125</td>
</tr>
<tr>
<td>TNF-α</td>
<td>F: 5'-ACGTAAGACAG&lt;br&gt;AACCCGCAAAG-3'&lt;br&gt;R: 5'-TGAAGAGAAACC&lt;br&gt;TGGGAGTAGAGAG-3'</td>
<td>150</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: CCACCTTTGAG&lt;br&gt;AGCTCATTTCT-3'&lt;br&gt;F: 5'-TCGTCTCCTCCTC&lt;br&gt;TGGTGCTTCT-3'</td>
<td>140</td>
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Table I. Primers.
significantly lower in the TGXTC group than in the model group (P<0.01). According to the Mankin score, 1-5 constitutes early stage of OA; 6-9, middle stage of OA; and 10-14, late stage of OA. The results suggest that the cartilage damage in the model group was in the middle stages of OA, while for the TGXTC group cartilage damage occurred in the early stages of OA (Fig. 1D).

**TGXTC reversed microstructural damage of subchondral bone.** In the sagittal plane of the subchondral bone, the trabecular bone was arranged in an orderly manner in the normal group (Fig. 2A). In the model group, the trabecular bone became broken and disordered (Fig. 2B). However, after treatment with TGXTC, trabecular bone was relatively regular (Fig. 2C).

In the cross section of subchondral bone, the trabecular bone had a uniform network in the normal group (Fig. 3A). In the model group, increased disorder of the trabecular bone was evident with a large osteophyte (Fig. 3B). However, after treatment, the structure of trabecular bone was improved and arranged neatly (Fig. 3C).

In the 3D models of each experimental group, tibial plateau became deformed with large osteophyte in the model group (Fig. 4B), while no osteophyte was present in the normal group (Fig. 4A). In the TGXTC group, the osteophyte was significantly decreased compared to the model group (Fig. 4C).
The mean and standard deviation values of the micro-CT parameters are shown in Fig. 5. The Tb.N, Tb.Th and BV/TV were increased while the Tb.Sp was decreased in the model group compared with the normal group (P<0.05 or P<0.01). However, Tb.N, Tb.Th and BV/TV were reduced in the TGXTC compared with the model group, whereas Tb.Sp in the TGXTC was increased compared to the model group (P<0.05 or P<0.01). These results suggest that subchondral sclerosis and microstructure damage in the model group, whereas TGXTC could improve structural injury of subchondral bone.

TGXTC decreased ALP and TRAP. ALP activity was significantly higher in the model than the normal group (P<0.01; Fig. 6A). After treatment, the TGXTC group exhibited a significant decrease in the ALP activity compared with the model group (P<0.01; Fig. 6A).

A similar trend was identified for TRAP activity (Fig. 6B). After inducing OA, TRAP activity was significantly higher in the model compared to the normal group (P<0.01). After treatment, the TGXTC group exhibited a significant decrease in TRAP activity compared to the model group (P<0.01).
**TGXTC decreased the OPG/RANKL ratio.** The ratio of OPG/RANKL was significantly higher in the model than that in the normal group (P<0.01; Fig. 6C). After treatment, the ratio of OPG/RANKL was significantly decreased in the TGXTC group compared to the model group (P<0.01; Fig. 6C).

**TGXTC inhibits the mRNA expression of IL-1β and TNF-α.** The mRNA expression of IL-1β was significantly higher in the model than that in the normal group (P<0.01; Fig. 7A). After treatment, the IL-1β mRNA expression was significantly decreased in the TGXTC group compared to the model group (P<0.01; Fig. 7A). mRNA expression of TNF-α was also significantly higher in the model compared to the normal group (P<0.01; Fig. 7B). After treatment, TNF-α mRNA expression was also significantly decreased in the TGXTC group compared to the model group (P<0.01; Fig. 7B).

**TGXTC inhibits the protein expression of IL-1β and TNF-α.** The protein expression of IL-1β was significantly higher in the model than the normal group (P<0.01; Fig. 8A and C). The protein expression of TNF-α was significantly higher in the model than the normal group (P<0.01; Fig. 8B and D). After treatment, the TNF-α protein expression was markedly decreased in the TGXTC compared with the model group (P<0.01; Fig. 8B and D).

**Discussion**

Although significant progress has been made in understanding OA pathophysiological pathways, much remains to be done to develop a specific therapy that could effectively retard or prevent progression of the disease. To this end, the literature suggests that in addition to cartilage, other articular tissues, including the subchondral bone, should be targeted. Previous findings have shown that TGXTC has therapeutic effects on knee OA (25) through multiple targets (22,23,25-27). In this study, we investigated the effect of TGXTC on subchondral bone and its relevant mechanisms of inflammatory factors.

To determine the effects of TGXTC on cartilage structure and the stage of OA, we used the Mankin score. In the model group, the articular cartilage structure was damaged; the stained proteoglycans became weaker, showing severe loss...
of proteoglycans. However, TGXTC treatment altered loss of proteoglycans and appearance of the cartilage structure, and the proteoglycan staining was improved compared to the normal group. The result shows that TGXTC improves cartilage matrix to promote increased production of proteoglycans. Additionally, the lower the modified Mankin score of the animal is, the better the outcome with respect to delaying the progression of OA (9). Therefore, we used Mankin scoring principles to analyze alteration of cartilage degeneration after TGXTC treatment. We found the Mankin score was decreased in the TGXTC group compared with the model group, which showed the stage of OA is from the middle stage to the early stage. These findings suggest that TGXTC has better therapeutic effects on mitigating cartilage damage and preventing the development of OA.

As the subchondral bone and cartilage are closely related, structural changes in the subchondral bone can further aggravate cartilage damage. Thus, we can delay the progression of OA by regulating bone remodeling to improve the subchondral bone structure (30,31). In the present study, the effect of TGXTC on subchondral bone was observed by micro-CT, which enables non-destructive assessment of the subchondral bone both quantitatively and qualitatively (10). We found osteophyte formation in the model group, with osteophytes narrowing the area in TGXTC group. In the model group, the increase of Tb.N and BV/TV indicate subchondral bone trabeculae undergo sclerosis. However, after treatment, the sclerosis was improved. We speculate the treatment with TGXTC could change these parameters by regulating bone remodeling, and the structure of subchondral bone was altered by improving the trabecular bone parameters.

Bone remodeling is a highly complex process by which old bone is replaced by new bone, in a cycle comprised of three phases: i) Initiation of bone resorption by osteoclasts, ii) the transition (or reversal period) from resorption to new bone formation, and iii) the bone formation by osteoblasts (32,33). ALP is essential for bone mineralization, which is secreted by osteoblast (34). Increased ALP level in serum has been observed in conditions such as rapid bone loss (35) and fracture risk (36,37). TRAP is secreted by osteoclasts during bone resorption, and serum TRAP activity correlates with resorptive activity in disorders of bone metabolism (14). In order to further explore the regulatory mechanism of the subchondral bone reconstruction, ALP and TRAP activity in serum was detected. In the present study a significant increase in ALP and TRAP levels was observed in the model group. These results indicated that a high bone formation and bone resorption of the bone remodeling process were both increased in OA. Subchondral sclerosis was observed using micro-CT; thus, we suggest that the rate of bone formation is greater than the rate of bone resorption. By contrast, treatment of TGXTC showed significantly decreased ALP and TRAP activity, suggesting that the potency of TGXTC is due to a decrease in the bone remodeling process by reducing bone formation and resorption to alleviate subchondral bone sclerosis in OA rabbits.

During OA, bone remodeling causes an imbalance between bone resorption and bone formation, leading to structural changes in the subchondral bone. Recently, the OPG/RANKL signaling pathway was shown to be involved in the regulation of osteoblastogenesis and osteoblast activity, which was regarded as a key factor in inhibiting bone proliferation and directly participates in the process of bone formation and bone resorption (38,39). Bone remodeling is also controlled by the balance of the OPG/RANKL ratio (18,40). Higher OPG/RANKL ratios mediate bone formation (41), while lower OPG/RANKL ratios mediate bone resorption (42). The OPG/RANKL production ratio was significantly increased in the model group, indicating that the remodeling rate of the subchondral bone was faster, excessive bone protection developed, and bone resorption was suppressed. However, following treatment with TGXTC, the OPG/RANKL ratio was significantly decreased, suggesting that TGXTC can reduce the remodeling rate by stabilizing bone resorption and bone formation to delay subchondral bone damage.

Previous studies proved that the regulation of bone metabolism occurs through the classical OPG/RANK/RANKL pathway, the direct and indirect functions of which are influenced by inflammatory factors, such as IL-1β, TNF-α, IL-6 and IL-17 (20,22,43-48). There are reports that TNF-α and IL-1β and other inflammatory factors can reduce OPG, stimulate RANKL and have a synergistic effect, resulting in increased bone resorption (47,49). In the present study, we found that the mRNA and protein expression of IL-1 and TNF-α in subchondral bone initially exhibited a significant increase in the model group and then decreased after treatment with TGXTC. These results indicated that inflammatory factors increased in OA, and TGXTC can decrease inflammatory factors to improve joint damage.

Taken together, results of the present study suggest that TGXTC could delay the pathological development of OA by regulating subchondral bone remodeling through regulation of the formation/resorption balance and its relating inflammatory factors. This may partly explain its clinical efficacy in the treatment of knee OA.

Acknowledgements

The authors would like to thank Professor Liu Xianxiang, from the Academy of Integrative Medicine, Fujian University of Traditional Chinese Medicine, for his helpful discussion.

Funding

This study was supported by the National Natural Science Foundation of China (grant no. 81774345) and the Developmental Fund of Chen Keji Integrative Medicine (grant no. CKJ2018003).

Availability of data and materials

The datasets used during the present study are available from the corresponding author on reasonable request.

Authors’ contributions

WC and SC conceived and designed the study, and reviewed and edited the manuscript. YH and MH performed the histology experiment. RL and ZL performed the micro-CT evaluation experiment. LZ performed the serum biochemistry assay experiment. JZ and GW performed the RT-qPCR experiment.
and the data analysis. XL and SC performed the western blot analysis experiment. GW designed the experiment and wrote the manuscript. All authors approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine (Fujian, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


