Protective effects of the Tougu Xiaotong capsule on morphology and osteoprotegerin/nuclear factor-κB ligand expression in rabbits with knee osteoarthritis

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Abstract. The imbalance of subchondral bone remodeling is a common pathological feature in the progression of osteoarthritis. In the current study, using a rabbit model of knee osteoarthritis, the effects of the Tougu Xiaotong capsule (TGXTC) on the cartilage and subchondral bone were investigated. In addition, osteoprotegerin (OPG), an inducer of bone formation, and receptor activator of nuclear factor-κB ligand (RANKL), a regulator of bone resorption in the subchondral bone, were assessed, in order to further explore the protective role of TGXTC in subchondral bone remodeling. The rabbit model of knee osteoarthritis, which was induced by a modified version of Hulth's method, was treated with TGXTC or glucosamine hydrochloride for 4 or 8 weeks. Subsequently, the tibia and femur were harvested for observation of cartilage histology, and the subchondral bone was observed by scanning electron microscopy. The expression levels of OPG and RANKL at the gene and protein levels were determined by reverse transcription-quantitative polymerase chain reaction and western blotting. TGXTC and glucosamine hydrochloride were identified to mitigate cartilage injury, reduce trabecular number and thickness and accelerate trabecular separation. It was additionally observed that the level of OPG mRNA and protein expression was reduced, and the RANKL mRNA and protein expression level was increased, in addition to the observation of a lower OPG/RANKL ratio in the TGXTC and hydrochloride groups. Taken together, these results suggest that TGXTC may mitigate cartilage injury and subchondral sclerosis, thus delaying the pathological development of osteoarthritis. This is suggested to be mediated partly through the reduction of OPG expression and increase of RANKL expression, which reduces the OPG/RANKL ratio, suppressing excessive bone formation.

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Introduction

Osteoarthritis (OA) is a degenerative joint disease that frequently affects middle-aged and elderly individuals (1). OA is characterized by mechanical abnormalities involving the degradation of joint tissues, including the articular cartilage, subchondral bone and the synovium (2). Although numerous previous studies have focused on the cartilage, the subchondral bone was recently identified to serve an important role in the development of OA (3-6). The mineral density of subchondral bone is reduced during the early onset of OA, and bone mass is increased by the late stage, along with the presence of subchondral sclerosis and osteophytes. In addition, a vicious cycle develops between structural alterations in the subchondral bone and cartilage injury, which are closely associated with OA progression. Therefore, the subchondral bone is notable target for the treatment of OA.

Structural alterations and abnormal bone remodeling of the subchondral bone are frequently present in patients with OA. The osteoprotegerin (OPG), receptor activator of nuclear factor-κB (RANK) and RANK ligand (RANKL) system is one of the most important molecular mechanisms that regulate bone remodeling (7). RANK is the receptor for RANKL, and OPG is a decoy receptor for RANKL. The OPG/RANKL ratio is crucial for the restoration of bone mass and repair of bone injury, due to the fact that it maintains the homeostasis between bone resorption and bone formation (8,9). Hence, delaying the pathological progression of OA by adjusting the expression of OPG and RANKL, in order to regulate the bone-remodeling rate, may lead to an improvement in bone microstructure.

Rofecoxib (a cyclooxygenase 2 inhibitor), ibuprofen and placebo are currently the standard treatment for OA (10), with the aim of reducing inflammation, controlling pain and providing the cartilage with the required nutrients. However, this approach involves the risk of adverse reactions, for example gastrointestinal ulcers (10). The Tougu Xiaotong capsule (TGXTC), characterized as a multi-target and multi-channel compound (11), contains a proven recipe for OA treatment, consisting of *Morinda officinalis*, *Paeonia lactiflora*, *Ligusticum chuanxiong* and *Sarcandra glabra* (12). Previous studies have demonstrated that this compound may inhibit chondrocyte apoptosis, promote chondrocyte proliferation (13-16), suppress expression of matrix metalloproteinases and inflammatory cytokines (17-19), improve the structure and function of cartilage (20) and promote osteoblast proliferation and activation (21). However, the regulatory effects of TGXTC on subchondral bone remodeling remain largely unclear. In the present study, the protective effects of TGXTC and glucosamine hydrochloride on the regulation of subchondral bone remodeling were compared, and the expression of OPG and RANKL were investigated in a rabbit model of knee OA, in order to explore the underlying mechanisms of TGXTC in OA treatment.

Materials and methods

Animals. A total of 72 female 6-month old New Zealand rabbits weighing 2.0±0.3 kg, were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) [license no. SCXK (Hu) 2012-0011]. These animals were raised in the Animal Center of Fujian University of Traditional Chinese Medicine, Fujian, China [license no. SYXK (Min) 2009-0001]. The care and use of the laboratory animals complied with the Guidance Suggestions for the Care and Use of Laboratory Animals, administered by the Ministry of Science and Technology (Beijing, China) (22).

Experimental design. Subsequent to one week of acclimation, the 72 rabbits were randomly divided into six groups, including the normal control, OA model, glucosamine hydrochloride (Bright Future Pharmaceuticals Factory Hong Kong, Yuen Long, Hong Kong, SAR, China), and low- (70 mg/kg/day), middle- (140 mg/kg/day) and high- (280 mg/kg/day) dose TGXTC (The Second People's Hospital of Fujian University of Traditional Chinese Medicine, Fuzhou, China; medical license no. MIN ZIZHI Z20100006) groups, with 12 rabbits in each group.

The rabbit model of OA was induced in all groups except for the normal control group using a modified version of Hulth's method (23). Rabbits were anesthetized by ear vein injection of sodium pentobarbital (3%; 30 mg/kg; Shanghai Xitang Biotechnology, Co., Ltd., Shanghai, China) and placed on an operating table in the supine position with 90˚C flexion of the right knee. The medial, collateral and anterior cruciate ligaments were transected via the medial approach, and the medial meniscus was removed. Successful transection was verified with the drawer test, and then the joint capsule and skin were sutured closed. Sodium penicillin (400,000 U; GE Healthcare Life Sciences, Logan, UT, USA) was administered intramuscularly for 3 consecutive days postoperatively. One week later, all animals were subjected to passive movement of the knee for 0.5 h daily for 4 weeks.

A total of five weeks postoperatively, the OA model was successfully established in the rabbits. Intragastric administration to the OA rabbits of glucosamine hydrochloride (75 mg/kg/day) and increasing doses of TGXTC (70, 140 and 280 mg/kg/day) was conducted either for 4 or 8 weeks, and an equivalent volume of normal saline was administered to those in the normal control or model groups.

Tissue collection. Following 4 or 8 weeks of the treatment, all animals were sacrificed with 2% pentobarbital sodium (40 mg/kg.wt via ear marginal vein injection; Merck Sharpe & Dohme, Shanghai, China) and the tibia and femur were collected for further investigation. The medial femoral condyle was prepared for histology and the tibia for scanning electron microscopy, and the subchondral bone isolated from the lateral femoral condyle was collected for the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis.

Histopathological examination. The femoral specimens were fixed with 4% paraformaldehyde (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 72 h, then decalcified with 10% EDTA (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) at room temperature for 16 weeks. Subsequently, the medial two-thirds of the medial femoral condyle was longitudinally cut into 1.2x1.2x0.5 cm sections and embedded in paraffin (Shanghai Guangkuo Chemical Co., Ltd., Shanghai, China). Finally, 4-μm thick sagittal sections were used for hematoxylin and eosin (H&E) staining and were observed under a light microscope (DM4000 B; Leica Microsystems GmbH, Wetzlar, Germany).

Scanning electron microscopy. Subsequent to fixation in 4% paraformaldehyde for 72 h, the medial two-thirds of the medial tibial condyle was sampled, rinsed with 0.1 M phosphate-buffered saline (GE Healthcare Life Sciences, Logan, UT, USA) in deionized water, dehydrated using tertiary butanol (Sinopharm Chemical Reagent Co., Ltd.), dried in a vacuum drier (Shanghai Jinghong Laboratory Instrument Co., Ltd., Shanghai, China), fixed onto the stage using conductive adhesive, then observed with a tabletop scanning microscope (TM3030; Hitachi, Ltd., Tokyo, Japan).

RT-qPCR. Total RNA was extracted from the subchondral bone of the lateral femoral condyle using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA) and quantified using a UV spectrophotometer (ND-2000C; Thermo Fisher Scientific, Waltham, MA, USA). cDNA (700 μg) was synthesized using the PrimeScript™ RT Reagent kit with gDNA Eraser (Takara Bio., Inc., Otsu, Japan). The PCR system was prepared according to the manufacturer's instructions, with 10 μl SYBR® Premix Ex *Taq* II (Takara Bio., Inc.), 0.4 μl ROX Reference Dye II (Takara Bio., Inc.), 0.8 μl upstream primer, 0.8 μl downstream primer, 2 μl cDNA and 6 μl dH_2O, with a total reaction volume of 20 μl. The PCR amplification protocol was as follows: Initial denaturation at 95˚C for 30 sec, followed by 40 cycles of denaturation at 95˚C for 3 sec and annealing at 60˚C for 30 sec (S1000; Bio-Rad Laboratories,

Inc., Hercules, CA, USA). The fluorescence signal of GAPDH acted as an internal reference for calculating the relative gene expression levels. RT-qPCR was performed using an 7500 Fast Real-Time PCR system (Applied Biosystems Life Technologies, Foster City, CA, USA). Primers were designed and synthesized by Takara Bio, Inc., and the sequences used are as follows: GAPDH, forward 5'-CCACTTTGTGAAGCT CATTTCT-3 and reverse 5'-TCGTCCTCCTCTGGTGCT CT-3; OPG, forward 5'-ACTACACAGACACTTGGCACA CC-3 and reverse 5'-CTTCCTCGCATTCACACACAC-3; RANKL, forward 5'-GCTAGGAGGGAGAGCAGCAA-3 and reverse 5'-TGAGAGAGGAAGACGGCACA-3.

Western blotting. The subchondral bone isolated from the lateral femoral condyle was immersed 1:10 in lysis buffer containing 50mM Tris (pH7.4), 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. The protein samples were electrophoresed by 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. Subsequently, the samples were incubated on a shaker at 4˚C overnight with the following primary antibodies: Mouse anti-β-actin (monoclonal; 1:5,000; cat. no. HC201; TransGen Biotech Co., Ltd., Beijing, China), rabbit anti-OPG (polyclonal; 1:1,000; cat. no. AV00033; Sigma-Aldrich, St. Louis, MO, USA) and rabbit-anti RANKL (polyclonal; 1:200; cat. no. BA1323; Boster Systems, Inc., Pleasanton, CA, USA). The samples were then rinsed with Tris-buffered saline with Tween-20 (TBST; Shanghai Jinghong Laboratory Instrument Co., Ltd.) and incubated with the following corresponding secondary antibodies: Goat anti-mouse horseradish peroxidase-conjugated IgG (monoclonal; 1:4,000; cat. no. HS201; TransGen Biotech, Inc). and goat anti-rabbit horseradish peroxidase-conjugated IgG (monoclonal, 1:4000; cat. no. HS101; TransGen Biotech, Inc.). This was performed on a shaker at room temperature for 1 h. Following incubation, the samples were rinsed with TBST, and developed using an enhanced chemiluminescence substrate (Beyotime Institute of Biotechnology). Image processing was conducted using scanning densitometry (170-8070 Molecular Imager ChemiDoc XRS System; Bio-Rad Laboratories, Inc.) to analyze gray values and to determine the relative expression of the proteins.

Statistical analysis. GraphPad Prism software, version 6.00 for Windows was used for statistical analysis. All quantitative data are expressed as the mean ± standard deviation. One-way analysis of variance was used, and $P<0.05$ was considered to indicate a statistically significant difference.

Results

TGXTC inhibits cartilage and subchondral bone degradation. In order to determine the protective effects of TGXTC on the morphology of cartilage and subchondral bone, the sections were evaluated by H&E staining. There was no evidence of degradation between cartilage and subchondral bone in the normal control group (Fig. 1Aa and Ba). However, the cartilage surface of the OA model group was partially damaged, with the disruption of the four-layer structure, disordered chondrocyte clusters and tidemark replication, in addition to the reduced staining intensity of the cartilage matrix (Fig. 1Ab and Bb). Subchondral sclerosis, involving increases in trabecular number and thickness and narrowing of the intertrabecular space was observed (Fig. 2A), suggesting the middle or late stages of OA.

Following 4 weeks of treatment, increased staining intensity of the cartilage matrix, reduced trabecular number and an increased intertrabecular space were observed in the glucosamine hydrochloride and TGXTC groups when compared with the OA model group, suggesting that glucosamine hydrochloride and TGXTC improve the morphology of cartilage and subchondral bone of the OA. Compared with the glucosamine hydrochloride group, increased the number of chondrocytes (Fig. 1Ac-f) and smooth or straight trabecular bone (Fig. 2B and C) were observed in the TGXTC groups, indicating that TGXTC may be more suitable for treating the OA model induced by a modified version of Hulth's method.

Subsequent to 8 weeks of treatment, more pronounced degradation of the cartilage and subchondral bone was observed in the OA model group. Although the cartilage surface appeared to contain fissures, extending deep into the radial layer in the glucosamine hydrochloride group, the structure of the cartilage and subchondral bone in this group appeared clearer than that of the OA model (Fig. 1Bc). Increased regular chondrocyte clusters and reduced tidemarks were observed in the TGXTC groups compared with the OA model and glucosamine hydrochloride groups, which is consistent with the results of the treatment for 4 weeks. Notably, the middle-dose TGXTC group exhibited greater improvement than the other doses, suggesting that the protective role of TGXTC was not dose-dependent.

TGXTC inhibits OPG expression and promotes RANKL expression. In order to further investigate the mechanism of TGXTC on subchondral bone remodeling, the expression levels of OPG and RANKL were analyzed using RT-qPCR and western blotting. A total of nine weeks subsequent to the induction of OA (4-week treatment group), the OPG mRNA and protein expression levels were significantly increased in the OA model group compared with the normal control group ($P<0.05$). A total of 13 weeks subsequent to the induction of OA (8-week treatment group), the mRNA and protein expression levels of OPG were not significantly different between the OA model and normal control groups, suggesting that increased OPG expression is only observed during the middle stage of OA progression.

Following 4 weeks of treatment, reduced OPG mRNA and protein expression levels were observed in the TGXTC and glucosamine hydrochloride groups, compared with the OA model group ($P<0.05$; Figs. 3A, 4A and C), suggesting that the excessive bone formation induced by OPG was inhibited by TGXTC and glucosamine hydrochloride.

The mRNA and the protein expression levels of RANKL in the OA model group were significantly lower ($P<0.05$)

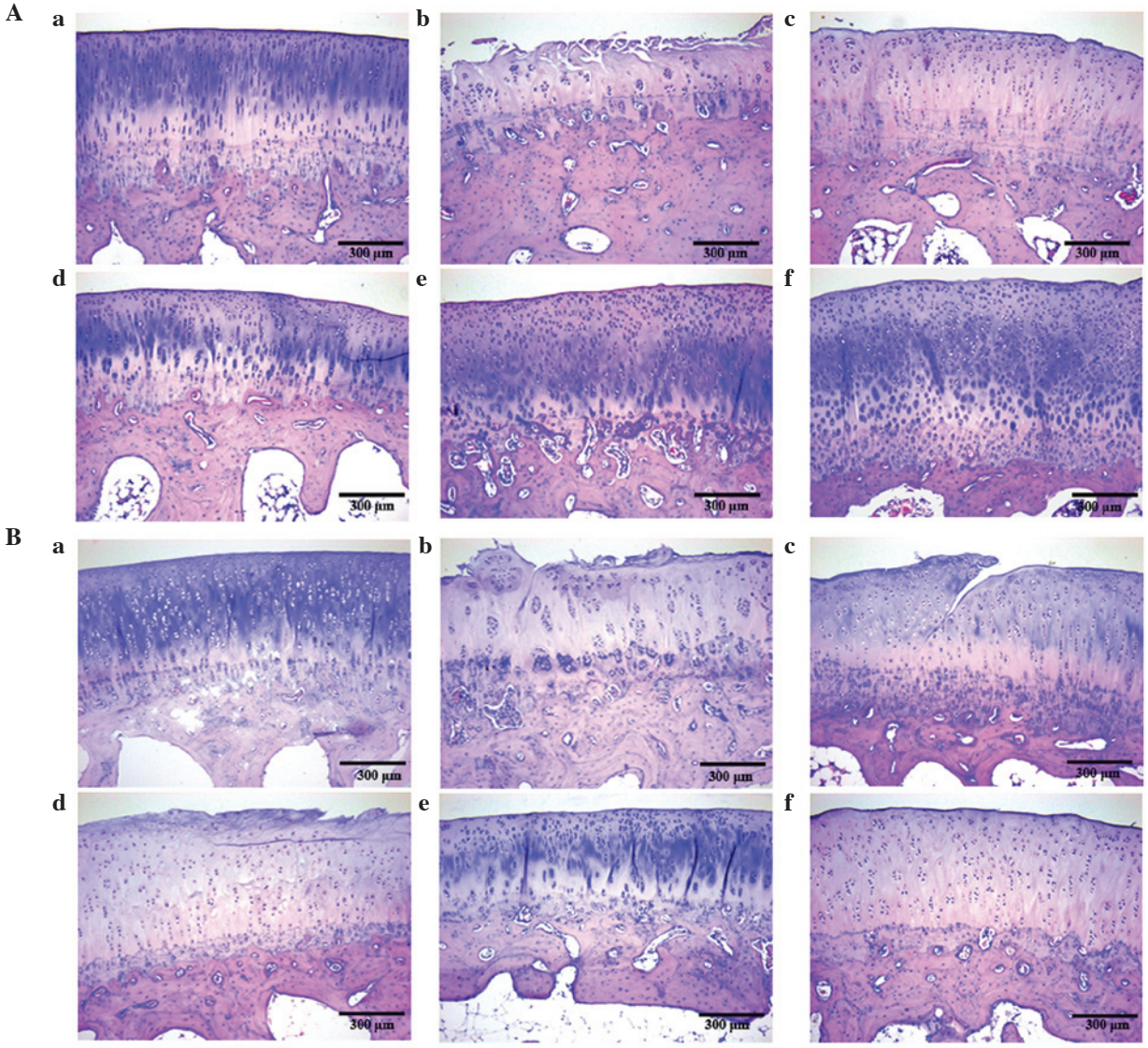

Figure 1. Cartilage microstructure of the medial femoral condyle following treatment for (A) 4 or (B) 8 weeks. Histopathological alterations were evaluated by hematoxylin and eosin staining and light microscopy at a magnification of x100. (a) Normal control, (b) osteoarthritis model, (c) glucosamine hydrochloride, (d) low-dose (70 mg/kg/day) TGXTC, (e) middle-dose (140 mg/kg/day) TGXTC and (f) high-dose (280 mg/kg/day) TGXTC groups. TGXTC, Tougu Xiaotong capsule.

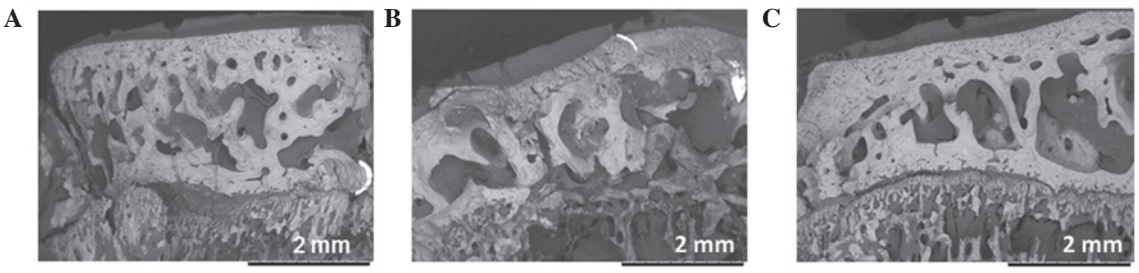

Figure 2. Fine structure of the subchondral bone of the medial tibial condyle following 4 weeks of treatment. The fine structure of the subchondral bone was observed by scanning electron microscopy, at a magnification of x30. (A) Osteoarthritis model, (B) glucosamine hydrochloride, (C) middle-dose (140 mg/kg/day) TGXTC. TGXTC, Tougu Xiaotong capsule.

than those in the normal control group subsequent to 9 weeks (4-week treatment group) or 13 weeks (8-week treatment group) of inducing OA, which indicates that insufficient bone resorption had occurred. Compared with the OA model group, a significant increase ($P<0.05$) in the RANKL mRNA and protein expression levels was observed between the glucosamine hydrochloride group and the TGXTC groups at 4 weeks. No significant difference in RANKL mRNA expression was observed between the glucosamine hydrochoride group and the OA group, however, the expression was increased in the

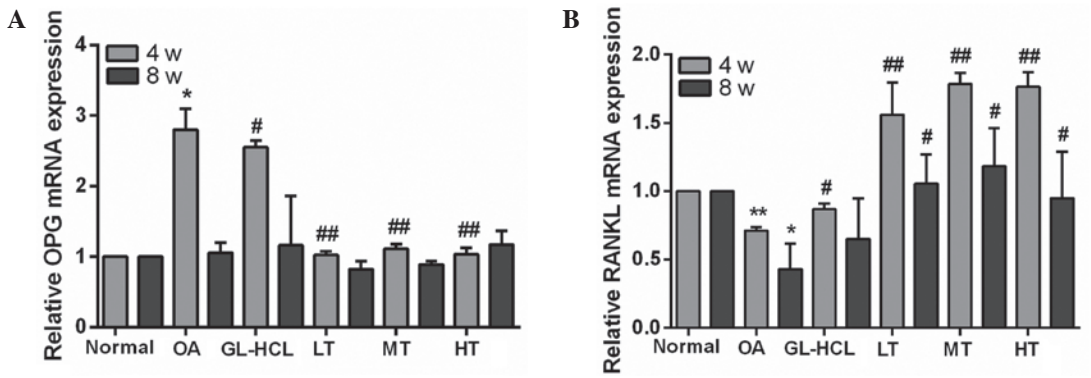

Figure 3. Relative mRNA expression of OPG and RANKL. The mRNA levels of (A) OPG and (B) RANKL were determined by reverse transcription-quantitative polymerase chain reaction. *P<0.05, **P<0.01 vs. normal control group. #P<0.05, ##P<0.01 vs. OA model group. OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-κB ligand; OA, osteoarthritis; GL-HCL, glucosamine hydrochloride; LT, low-dose (70 mg/kg/day) Tougu Xiaotong capsule group; MT, middle-dose (140 mg/kg/day) Tougu Xiaotong capsule group; HT, high-dose (280 mg/kg/day) Tougu Xiaotong capsule group.

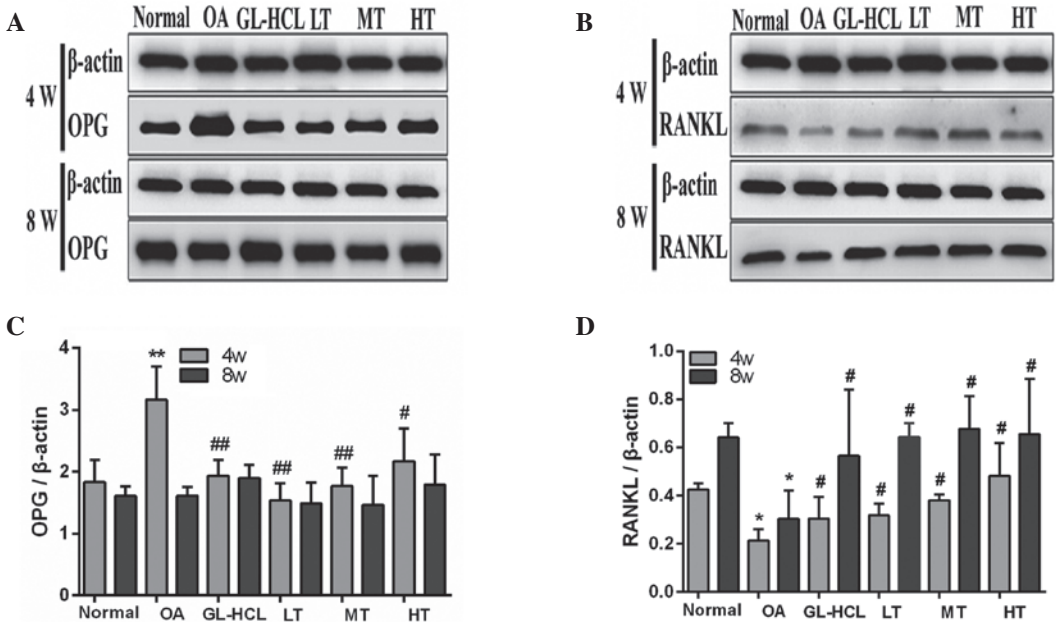

Figure 4. The protein expression of OPG and RANKL. The protein expression of OPG and RANKL were determined by the western blot assay. Chemiluminescent imaging for (A) OPG protein and (B) RANKL. Relative expression of (C) OPG and (D) RANKL. *P<0.05, **P<0.01 vs. normal control group. #P<0.05, ##P<0.01 vs. OA model group. OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-κB ligand; OA, osteoarthritis; GL-HCL, glucosamine hydrochloride; TGXTC, Tougu Xiaotong capsule; LT, low-dose (70 mg/kg/day) TGXTC group; MT, middle-dose (140 mg/kg/day) TGXTC group; HT, high-dose (280 mg/kg/day) TGXTC group.

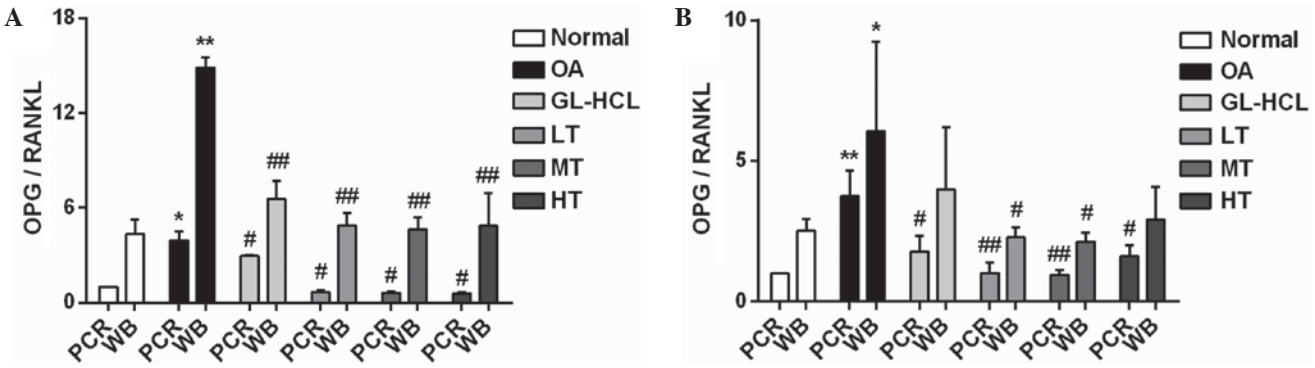

Figure 5. OPG/RANKL ratio (A) 4 and (B) 8 weeks of treatment. The OPG/RANKL ratio was used to determine the protective effect of TGXTC on subchondral bone remodeling. *P<0.05, **P<0.01 vs. normal control group. #P<0.05, ##P<0.01 vs. OA model group. OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-κB ligand; OA, osteoarthritis; GL-HCL, glucosamine hydrochloride; TGXTC, Tougu Xiaotong capsule; LT, low-dose (70 mg/kg/day) TGXTC group; MT, middle-dose (140 mg/kg/day) TGXTC group; HT, high-dose (280 mg/kg/day) TGXTC group; PCR, polymerase chain reaction; WB, western blotting.

TGXTC group at 8 weeks, compared with the OA model group. In addition, a significant increase (P<0.05) in RANKL protein expression levels was observed in the glucosamine hydrochloride group and the TGXTC groups at 8 weeks, compared with the OA model group (Figs. 3B, 4B and D). This suggests that TGXTC and glucosamine hydrochloride may selectively promote bone resorption through inducing the expression of RANKL.

TGXTC inhibits the OPG/RANKL ratio. To further determine the regulation of TGXTC on the homeostasis between bone resorption and bone formation, the OPG/RANKL ratio was analyzed for mRNA and protein expression. The OPG/RANKL ratio for the mRNA and the protein expression levels in the OA model group were significantly higher than those in the normal control group following 9 (P<0.05) or 13 (P<0.05) weeks of OA induction. This indicated that an imbalance of bone metabolism was involved in OA progression. Following 4 weeks of treatment, the OPG/RANKL ratio was significantly inhibited by the addition of glucosamine hydrochloride and TGXTC (P<0.05), compared with the OA model group (Fig. 5A). Following 8 weeks of treatment, similar results to the 4 weeks treatment group were observed in the mRNA (P<0.05) and protein expression levels of the low or medium TGXTC dose groups (P<0.05), however no reduction was observed in the protein level of the glucosamine hydrochloride group and in the high TGXTC dose group. This suggests that TGXTC may be more suitable for regulating the homeostasis between bone resorption and bone formation induced by the OPG/RANKL pathway in the late stage of OA, and that this protective effect is not dose-dependent.

Discussion

TGXTC, a traditional Chinese medicine, has been demonstrated to be clinically effective in the treatment of OA, which has been indicated by *in vitro* and *in vivo* studies where TGXTC was observed to reverse cartilage degeneration in OA (11-20). However, whether TGXTC has a protective effect on subchondral bone remodeling remains unclear. In the current study, TGXTC was demonstrated to be able to efficiently inhibit the imbalance of subchondral bone remodeling of OA, via the OPG/RANKL pathway.

OA progression involves various pathological alterations, including those involving cartilage, subchondral bone and the synovial membrane, while the degeneration of cartilage is the most typical characteristic of this disease (24). In the present study, multiple pathological alterations of the cartilage, containing those of the four-layer structure, cell number and arrangement, tidemarks, in addition to matrix staining were clearly observed in the OA model group, which indicates the incidence of OA. Following treatment with glucosamine hydrochloride and TGXTC, all of these pathological alterations were observed to be alleviated. In addition, the therapeutic effects of TGXTC are suggested to be preferable to those of glucosamine hydrochloride, indicating the benefits of treatment with TGXTC.

In addition to those in the cartilage, structural alterations in the subchondral bone can further aggravate the progression of OA. Thus, regulating subchondral bone remodeling may improve the subchondral bone structure, which would be beneficial by delaying the OA progression (25,26). The OPG/RANKL/RANK system is one of the most critical molecular mechanisms underlying the regulation of bone remodeling, and additionally serves an important role in maintaining the OPG/RANKL balance in bone remodeling (7). RANKL is secreted by osteoblasts and acts as a strong regulator of bone resorption. RANKL binds to its receptor, RANK, on osteoclast precursor cell, which induces osteoclast maturation, thereby mediating bone resorption (27). OPG, which is secreted by osteoblasts and bone marrow stromal cells, is essential for preventing bone resorption, and is a decoy receptor for RANKL (7). By binding to RANKL, OPG inhibits osteoclast proliferation and differentiation, reduces the production of mature osteoclasts and reduces bone resorption (28). Additionally, bone remodeling is controlled by the balance of the OPG/RANKL ratio (7,29), with a higher OPG/RANKL ratio mediating bone resorption (30) and a lower OPG/RANKL ratio mediating bone formation (31).

Abnormal bone remodeling during OA results in an imbalance between bone resorption and bone formation, leading to structural alterations in the subchondral bone. A total of 9 weeks subsequent to the induction of OA, OPG production was increased, RANKL production was reduced and the OPG/RANKL production ratio was significantly increased in the OA model group. This suggested that OPG expression reached a peak during the middle stage of OA, and the compensatory remodeling of the subchondral bone was faster. Although the increase in OPG levels was inhibited by compensatory remodeling following 13 weeks of OA induction, levels of RANKL were inhibited during the progression of OA. In addition, a higher OPG/RANKL ratio was observed in the OA model group, suggesting that there was continuous bone formation, which may be due to the subchondral sclerosis occurring during the late stages of OA.

Subsequent to 4 weeks of TGXTC treatment, OPG expression was reduced, RANKL expression was increased and the OPG/RANKL ratio was significantly reduced to levels similar to those of the normal control group. However, in addition to those treated with glucosamine hydrochloride or high doses of TGXTC, these effects were also observed in the low and medium TGXTC dose groups during following 8 weeks of treatment. This indicates that TGXTC may reduce the remodeling rate and stabilize bone remodeling to delay subchondral sclerosis with the appropriate dose, thus may be preferable for use in the regulation of subchondral bone remodeling in OA.

Taken together, these results suggest that TGXTC may alleviate damage to the cartilage and subchondral bone, and balance subchondral bone remodeling, delaying subchondral sclerosis via the regulation of OPG and RANKL expression. This may provide a novel therapeutic strategy for use in the treatment of OA.

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