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The systemic bone protective effects of Gushukang granules in ovariectomized mice by inhibiting osteoclastogenesis and stimulating osteoblastogenesis

Qiang Wang [a, b, d, 1], Yongjian Zhao [a, b, d, 1], Nannan Sha [a, b, d], Yan Zhang [a, b, d], Chenguang Li [a, b, d], Hao Zhang [a, b, d], Dezhi Tang [a, b, d], Sheng Lu [a, b, d], Qi Shi [a, b, d], Yongjun Wang [a, b, c, d], Bing Shu [a, b, d, **], Dongfeng Zhao [a, b, d, *]

[a] Longhua Hospital, China
[b] Spine Institute, China
[c] School of Rehabilitation Science, Shanghai University of Traditional Chinese Medicine, Shanghai, 200032, China
[d] Key Laboratory of Theory and Therapy of Muscles and Bones, Ministry of Education, Shanghai, 200032, China

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ABSTRACT

Primary osteoporosis (POP), which is caused by unbalanced bone remodeling, leads to significant economic and societal burdens globally. Gushukang (GSK) granule serves as one commonly used prescription for POP in Traditional Chinese Medicine (TCM). The present study aimed to clarify the exact roles of GSK in bone remodeling with *in vivo* and *in vitro* assays. Here we showed that GSK prevented bone loss and the alternations of osteoporotic bone parameters as well as the decreased density of osteoclast in ovariectomized (OVX) mice. GSK inhibited receptor activator for nuclear factor-κ B Ligand (RANKL)-activated osteoclastogenesis in bone marrow macrophages (BMMs). At the molecular levels, GSK inhibited the expression of nuclear factor of activated T cells cytoplasm 1(NFATc1) and c-Fos, two master regulators of osteoclastogenesis. GSK also inhibited bone resorbed genetic expression of matrix metalloproteinase 9 (*MMP9*), cathepsin K (*Ctsk*), *TRAP* and carbonic anhydrase II (*Car2*). Meanwhile, GSK stimulated osteoblastogenesis from bone primary mesenchymal stem cells (MSCs) and enhanced the expression of Osteirx, and Runx2. GSK also stimulated the expression of *Col-1*, *Osteocalcein* and alkaline phosphatase (*ALP*). Our investigation established the systemic bone protective effects of GSK by suppressing osteoclastogenesis and stimulating osteoblastogenesis and laid bases for new drugs discovery in treating POP.

Introduction

Primary osteoporosis (POP) causes bone fragility and an increased vulnerability to fractures, which causes significant economic and societal burdens globally.[1,2] The osteoclastic bone resorption that overpasses osteoblastic bone formation is the fundamental pathogenesis in POP.[3,4] Therefore, drugs that can inhibit osteoclastic bone resorption or stimulate osteoblastic bone formation are considered as two crucial methods in the managements of POP. Currently, there are several major drugs on the market[5–9]: estrogen,[5] selective estrogen receptor modulators,[6] bisphosphonates,[7] calcitonin[8] and parathyroid hormone.[1–34] However, these drugs target osteoblastic bone formation or osteoclastic bone resorption only and nearly no drugs targeting the both,[10] which will effectively reduce the bone loss as well as with fewer side effects. Therefore, it is necessary to find new effective drugs to treat POP in pursuit of better effect.

Herbal medicines have been prescribed in treating POP for long time and numerous investigation have realized that herbal medicine (Including natural compounds or marketed

* Corresponding author.
** Corresponding author.
E-mail addresses: siren17721101@163.com (B. Shu), zhaodongfeng0106@163.com (D. Zhao).
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[1] Contributed equally.

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prescriptions) have been reported to plays anti-inflammatory,[11] such as Tumor Necrosis Factor-α (TNF-α)-activated signaling,[12] and bone protective effect.[13,14] These finding indicate that both the bone formation and bone resorption are affected. Tonifying Shen (Kidney) principle in Traditional Chinese Medicine (TCM) theory represents one alternative and complementary method in treating POP[15] and Gushukang (GSK) granule currently serves as one of the most used prescription. Previous investigation have well established that the intervention of GSK granule has been reported to increase bone mineral density (BMD) in hydrocortisone-induced bone loss in Wister rats[16] and bone loss in type 1 diabetes.[17] Moreover, following investigation with effective component of GSK granule, icariin, has found that it stimulates osteoblastogenesis.[18] However, both the direct and indirect effects of GSK granule on osteoclastogenesis are rare investigated. Therefore, it is necessary to investigate the bone protective effects of GSK granule in treating POP as well as its underlying mechanism.

Bone tissue undergoing constant dynamical remodeling process to guarantee normal bone mineral density (BMD) and function.[19] This process requires the balanced remodeling process between bone formation and bone resorption.[20] On one hand, osteoclasts differentiate from bone marrow macrophages (BMMs) by the stimulation of macrophage colony stimulating factor (M-CSF) and receptor activator for nuclear factor-κ B Ligand (RANKL).[21,22] RANKL up-regulates the expression of crucial makers to activate osteoclastogenesis, such as nuclear factor of activated T-cells-cytoplasm 1(NFATc1)[23] and c-Fos.[24] Moreover, RANKL activates the expression of bone resorption encoding genes, such as matrix metalloproteinase 9 (MMP9), cathepsin K (Ctsk), tartrate-resistant acid phosphate (TRAP) and carbonic anhydrase II (Car2).[25] On the other hand, osteoblasts differentiate from bone mesenchymal stem cells (MSCs) by the stimulation of many factors, such as the expression of runt-related transcription factor 2 (Runx2), and Osterix.[26] Collectively, these factors up-regulate the expression of alkaline phosphatase (ALP), Col-1, and Osteocalcein (OCN), which are crucial markers for the formation of functional osteoblasts[27] and bone formation.

In this study, we performed *in vitro* study with OVX mice to assess bone protect effects of GSK granule on POP. Moreover, we carried out *in vitro* assay to fully reveal the indirect and direct systematic regulatory effects of GSK on osteoclastogenesis and osteoblastogenesis.

Materials and methods

Materials

GSK was provided by Kangchen Company (Liaoning, China; Cat: Z2003255). The herbal contents were shown in Supplemental Table 1 and the marker substance (10 representative effective components) with HPLC assays was shown in Supplemental Table 2). Caltrate (600mg Calcium carbonate and 125IU Vitamin D3/Tablet) were commercial purchased from Huishi Company (Jiansu, China). Dexamethasone, and β-Glycerophosphate were purchased from Sigma (Nowra, NSW, Australia) and M-CSF and RANKL were purchased from Peprotech (New Jersey, USA). Primary antibodies against Osterix, OCN were purchased from Cell Signaling Technology (Beverly, MA, USA) and NFATc1, and c-Fos was purchased from Santa Cruz Biotechnology (CA, USA). The primers of *MMP9, Ctsk, TRAP, Car2, NFATc1, c-Fos, Col-1, ALP, OCN, Runx2* and *ß-actin* were synthesized by Shenggong Company (Shanghai, China), and the primer sequences were listed in Table 1.

Table 1
Names and sequences of primers used for polymerase chain reaction analysis.

Gene	Sequence
ß-actin	F: 5′-CCTGTACGCCAACACAGTGC-3′
	R:5′- ATACTCCTGCTTGCTGATCC-3′
OCN	F:5′- CTTGAAGACCGCCTACAAAC-3′
	R:5′- GCTGCTGTGACATCCATAC-3′
Col-1	F:5′- GCACGAGTCACACCGGAAC-3′
	R:5′- CCAATGTCCAAGGGAGCCAC-3′
ALP	F:5′- GAATCAAATGTTCAGGGTGGT-3′
	R:5′- TGGCACGTTAAAGGTAATCAG-3′
Runx2	F:5′-CTCTTCTGGAGCCGTTTATGT-3′
	R 5′- GTTTCTTAGGGTCTTGGAGTGA-3′
MMP9	F: 5′- CTTCTTCTCTGGACGTCAAATG -3′
	R:5′-CATTTTGGAAACTCACACGCC -3′
Car2	F:5′- AGAGAACTGGCACAAGGACTT -3′
	R 5′- CCTCCTTTCAGCACTGCATTGT -3′
Ctsk	F:5′- GATGCTTACCCATATGTGGGC -3′
	R:5′- CATATCCTTTGTTTCCCCAGC -3′
TRAP	F:5′- GCCAAGATGGATTCATGGGTGG -3′
	R:5′- CAGAGACATGATGAAGTCAGCG-3′
NFATc1	F:5′- TGTTCTTCCTCCCGATGTCT -3′
	R:5′- CCCGTTGCTTCCAGAAAATA -3′
c-Fos	F:5′- TGTTCTTCCTCCCGATGTCT -3′
	R:5′-GGATTTGACTGGAGGTCTGC -3′

OCN, osteocalcein; *Col-1*, collagen1; *ALP*, alkaline phosphatase; *Runx2*, runt-related transcription factor 2; *Car2*, carbonic anhydrase II; *Ctsk*, cathepsin K; *Trap*, tartrate-resistant acid phosphatase; *Mmp9*, matrix metalloproteinase 9; *NFATc1*, nuclear factor of activated T-cells c1; *c-Fos*, FBJ osteosarcoma oncogene; F, forward; R, reverse.

Animal treatments

Three-month-old female C57BL/6 mice (SLAC Laboratory Animal Co. Ltd., Shanghai, China) were maintained in animal center of Longhua hospital with light: dark (12 h:12 h) condition and fed with commercial diet and distilled water ad libitum. The mice were divided into 6 groups after ovariectomy: control (sham + saline), OVX (OVX + saline), CAL (OVX + Caltrate), GSKL (OVX + low dose GSK granule, 2 g/kg/day), GSKM (OVX + medium dose GSK granule, 4 g/kg/day) and GSKH (OVX + high dose GSK granule, 8 g/kg/day). Mice in GSKL, GSKM and GSKH groups were intragastrically administered three different dosages of GSK granule in saline based on the Meeh–Rubner equation. CAL group were treated with Caltrate dissolved in saline (0.372 tablet/kg/day). Mice in control group and OVX group were treated with equal amounts of saline. Samples were collected for histological analysis at the end of 3-month intervention. All of the experimental protocols were performed with the approval of Institutional Animal Care and Use Committee of the Shanghai University of TCM.

Micro-CT analysis

Left tibias in each group were fixed in 10% neutral buffered formalin (Wexis, Guangzhou, China) for 24 h, and washed for 2 h with tap water. The μCT analyses were carried out using μCT80 radiograph microtomography (Scanco Medical AG, Switzerland). Briefly, the scanner parameters were set as follow: voltage 55 kV, current 72 μA and voxelsize 10 μm. Three-dimensional (3D) model reconstructions (threshold 245–1000) of the proximal tibia (1 mm) were carried out with Ray V3.4A model visualization software (Scanco Medical AG, Switzerland) under the growth plate. The bone volume (BV) of astragalus was measured and a density threshold was set from 370 to 1000 as bone and a stack of 340–441 cross sections were performed. Consequently, the following parameters including BMD, ratio of BV to tissue volume (BV/TV), trabecular bone number (Tb.N), trabecular bone thickness (Tb.Th), and

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