Zuogui Wan alleviates ovariectomy-induced osteoporosis by maintaining FoxO3 and increasing NK1R

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Introduction

Postmenopausal osteoporosis (PMOP) is a kind of common disease associated with aging, mainly in postmenopausal women. Bone loss caused by the lack of estrogen further changes the bone structure aggravates osteoporosis, is easy to fracture, seriously affects the health and quality of life of the elderly, and even shortens life expectancy(1-3). For long-term osteoporosis treatment, sequential use of a bone-forming agent and an antiresorptive agent has been shown to provide better clinical outcomes(4). Osteogenic drugs include parathyroid hormone, prostaglandin E2, calcium and vitamin D. Drugs that inhibit osteoclasts include estrogen replacement therapy and bisphosphonates(5, 6). These drugs act directly on the processes of bone formation and absorption and mainly inhibit osteoclasts. Of course, these drugs play an important role in the prevention and treatment of osteoporosis, but their use is limited by high treatment costs and side effects such as cancer, stroke and heart disease. Therefore, the search for drugs with superior efficacy and fewer side effects is still a problem to be solved.

Traditional Chinese medicine (TCM) has been used to treat various diseases for thousands of years because of its stable efficacy, few side effects and low price. Zuoguiwan (ZGW), a typical Chinese herbal medicine, which was formulated by Zhang Jingyue, a famous physician in Ming Dynasty and first recorded in Jingyue Quanshu, could be used to strengthen bone(7, 8). With the development of modern medicine, the mechanism of ZGW for bone strengthening has been gradually recognized. ZGW plays critical roles in chronic kidney disease-mineral and bone disorders by adjusting the metabolism of calcium and phosphorus(9, 10). In addition, a number of studies have shown that ZGW has no acute, chronic toxicity and other side effects on the liver and kidney, demonstrating the therapeutic safety of ZGW in clinical application(11, 12). In our previous experiments, a series of experiments proved that ZGW up-regulated the level of OPG, down-regulated the expression of RANKL, increased the expression of Runx2 mRNA, and promoted the proliferation of BMSCs in vitro(13). In terms of mechanism exploration, our previous study also showed that ZGW has a bone protective effect and regulates bone metabolism imbalance in oophrectomized rats through β2AR mediated RANKL/OPG(13). However, the mechanism of ZGW in osteoporosis has not been adequately study.

Neurokinin-1 receptor (NK1R) is a member of the Neurokinin receptors family(14, 15). As the receptor of Tachykinins, it is a member of the family that interacts most closely with SP(16). When SP binds to the NK1R ligand, they internalize the nucleating endosome and induce the grid-dependent internalization of the receptor. After that, SP is degraded and NK1R is recovered to the cell surface(17). NK1R is expressed by osteoblast, osteoclasts, and their precursors. Neuropeptides such as SP can bind to receptors and activate NK1R to control the function of osteoclasts and osteoblasts and participate in bone repair, reconstruction and growth by binding to receptors and activating NK1R(18). Over the last few years, the func-
tion of SP has been demonstrated in different osteoporosis models. Reduction in SP was observed in both ovariec-
tomized female rats and in sciatic nerve-resected rats(17, 19). In addition, SP blocked bone microstructure damage and bone mineral density reduction induced by ovariec-
tomy in rats, suggesting its therapeutic effect on osteopo-
rosis. However, whether ZGW has an effect on SP/NK1R and its mechanism have not been explored.

We observed low NK1R expression in BMSC cells extracted from OVX rats. The level of NK1R in BMSC cells from OVX rats treated with ZGW was rescued. Fur-
thermore, ZGW also increased the level of FoxO3 in cells, where FoxO3 was identified as a transcription factor of NK1R. Our findings indicate that FoxO3/NK1R is vital for Signaling pathways in the treatment of osteoporosis. ZGW is also a potential drug for the treatment of osteoporosis.

Materials and Methods

Reagents

Primary antibodies against NK1R (ab183713), OSX (ab209484) and PPAR γ (ab272718) were purchased from Abcam (USA); FoxO3 (9532S), as well as secondary antibody (12829s) were purchased from Cell Signalling Technology (USA). Glyceraldehyde 3-phosphate dehy-
drogenase (GAPDH, GB11002) was acquired from Ser-
vicebio (Wuhan, China). The Dual Luciferase Assay Kit was acquired from Promega (E1910, Promega, US). All reagents were used according to the instructions.

Animal models and drug administration

Sixty Sprague-Dawley female rats were randomly divi-
ded into six groups and given normal saline, OVX group (1mL/100g/d, OVX), alendronate treatment group (63mg/
kg/d, OVX-A), ZGW low dose [2.42g/kg/d freeze-dried powder, OVX-ZGW(L)], ZGW medium dose [4.84g/kg/d freeze-dried powder, OVX-ZGW(M)] and ZGW high dose [9.68g/kg/d freeze-dried powder, OVX-ZGW(H)]. Sham operation rats were given normal saline placebo (1mL/100g/d, Sham). All the animals were raised in the Animal Center of Nanjing University of Chinese Medic-
e. The conditions were as follows: four in each cage, free feeding and drinking, 12h day-night cycle, room tem-
perature 22.6±0.4℃, After 12 weeks of administration, the rats were anesthetized with 3% pentobarbital solution (0.2mL/100g). After blood collection, the rats were sacri-
ficed and the bilateral femurs and tibia of the hippocam-
pus were taken for further study. All animal experimental designs and plans have been approved and approved by the Animal Ethics Committee of Nanjing University of Chinese Medicine (Ethics No. 201901A011).

Quantitative real-time PCR assay

Total RNA was extracted according to the commercial instructions for the Tissue/Cell RNA Rapid Extraction Kit. According to the commercial protocol of HiFaiTM 1 Strand cDNA Synthesis SuperMix, cDNA was synthesized for qPCR. QPCR was performed on the cDNA samples by real-time PCR (ABI 7500, Applied Biosystems, Inc., Waltham, MA, USA) in accordance with commercial instruc-
tions Hieff™ qPCR SYBR Green master mix, with the amplification procedure of 1 cycle for 95 °C for 5 min, followed by 40 cycles of 10 s of denaturation at 95 °C, 20 s of annealing at 60 °C, 20 s of extension at 72 °C, and then followed 1 cycle, with the three stages of dissolution curve for 15 s at 95 °C, 60 s at 60 °C, and 15 s at 95 °C. Pri-
mer sequences were purchased from Realgene, Nanjing. GAPDH was used as an internal control. Primers are pur-
chased from China Shenggong Co., LTD. Data were ana-
yzed and calculated using the 2△△Ct technique.

Western blot

Total protein was extracted with a protein extraction buffer. Each sample was separated by 8%-10% SDS-PAGE for about 20 µg. The sample was then transferred to a 0.45 mm polyvinylidene fluoride membrane. After blocking with 5% BSA solution for 1 h, the bands were incubated with the first antibody overnight. The bands were cultured with secondary antibodies and were Goat anti-mouse IgG (H+L) (1:10000) or Goat anti-rabbit IgG (H+L) (1:10000). The films are visualized using Enhanced ECL Chemilumi-
nescent Substrate Kit and then exposed to X films by gel recording system (Image Quant LAS 4000, GE Co., Ltd, Boston, MA, USA). Image J software (National Institutes of Health, Bethesda, MD, USA) detects and analyzes the total density of the membrane.

Alizarin Red staining

BMSCs were immobilized in 4% PFA for 20 min at room temperature, then rinsed with 1 PBS and ddH2O. Then it was stained with 2% alizarin red staining (Sigma-
Aldrich) for 10 minutes, washed with water, fixed with xyl-
elene and imaged. For quantification, ImageJ 1.55(National Institutes of Health) was used to calculate the alizarin red reaction area (shown in arbitrary units) for each amplifi-
cation field, and the average was repeated for individual organisms.

Luciferase reporter gene assays

A dual-luciferase reporter assay was employed to exa-
mine the interaction between NK1R and FO XO. The
NK1R promoter (−2000 to +100, −700 to +100, −200 to
+100) was amplified and cloned into the pGL3 basic vec-
tor (Promega, USA). HEK 293T cells (5 × 104 cells per
well in 96-well plates) were co-transfected with NK1R
promoter construct plasmids and FOXO3 overexpression plasmids. After 48 h of transfection, the HEK 293T cells were lysed and subjected to luciferase activity measure-
ment with a Dual-Luciferase Assay Kit (E1910, Promega, US) according to the manufacturer's instructions.

CHIP-PCR

The cells were treated with formaldehyde, and the cells
were collected and broken by ultrasound. Antibodies to
FOXO3 were added, and the target protein-DNA complex was bound overnight. Protein A was added to bind the antibody-DNA complex and precipitated. The precipitated complexes were washed to remove some non-specific binding. After elution, an enriched target protein-DNA complex was obtained. Finally, the enriched DNA fragments were purified for PCR analysis.

Statistical analyses

All data were expressed as means ± standard deviation. One-way analysis of variance followed by post hoc analy-
sis using the Student-Newman-Keuls test (more than two groups), and Student's t-test (two-group comparison) were used to calculate and analyze all results. Statistical ana-
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Results

NK1R decreased in BMSCs derived from OVX rats

In the previous literature and our study, it has been proved that ovariectomized rats can lead to secondary osteoporosis. We assayed the endogenous expression level of relative protein and mRNA in BMSCs from OVX rats. Compared with the sham group, BMSCs from OVX rats showed a marked decreased in NK1R expression (Fig. 1A). At the same time, OSX, the osteogenic differentiation-specific factor, was significantly decreased, while PPAR γ, the lipid-specific factor, was significantly increased. The quantification of each protein is shown in Figure 1B. RT-PCR was also used to detect mRNA levels of NK1R, OSX and PPAR γ, and the results were consistent with the protein results (Fig. 1C).

ZGW eliminated the decrease in NK1R induced by OVX

In order to explore the effect of ZGW on OVX-induced osteoporosis and the underlining mechanisms, we respectively used positive drug alendronate, low, medium and high dose ZGW to feed OVX-induced rats. As shown in Figure 2A, ovarian removal leads to a reduction in NK1R, which can be ameliorated by the positive drug Alendronate, and the recovery of NK1R by ZGW is also dose-dependent. The same trend was observed in OSX, but the effect was not as obvious as in NK1R. PPAR increased by OVX can also be inhibited by high doses of ZGW. The quantitative analysis of each protein is shown in Figure 2B.

ZGW ameliorated osteoporosis caused by OVX

In previous studies, micro-CT was used to analyze the structural thickness of the distal femur of rats. In two-dimensional reconstruction sections, ZGW can significantly prevent OVX-induced bone loss and improve the microstructure of bone trabecular (13). In this experiment, BMDS were co-cultured with a drug-containing serum of experimental rats, and the effect of serum on cell calcification was observed under the induction of osteogenic medium for 2 weeks. Alizarin red staining results are shown in Figure 3A. The calcification alleviation effects of OVX were reversed by ZGW, as well as Alendronate. The higher the concentration of the drug, the more pronounced the effect. The quantitative analysis is shown in Figure 3B.
FOXO3 binds to the promoter of NK1R to increase its expression

To determine the presence of conserved regulatory elements in the NK1R promoter, we sequenced the promoter regions of these genes using Clustal Omega software and determined the predicted binding site for FOXO3 using Genomatix software. This analysis revealed three FOXO3 sites consistent with the NK1R promoter (Fig. 4A). To identify the NK1R promoter region that mediates FOXO3 activation of NK1R expression, we generated plasmids expressing luciferase reporter genes driven by full-length NK1R promoter and NK1R promoter fragments (-2000/+100 bp, -700/+100 bp, and -200/+100 bp). FOXO3 increased luciferase activity of the first two fragments (Fig. 4B). Chip qPCR further demonstrated that the −700/+100 bp fragment of the NK1R promoter contained a strong regulatory element that mediated FOXO3 activation (Fig. 4C). Subsequently, we found that OVX induced a decrease in FOXO3 levels, but this was offset by high dose of ZGW (Figure 4D). The mRNA level of FOXO3 induced by OVX was also recovered with ZGW (Figure 4E).

FOXO3 overexpression increased the expression of NK1R

To verify whether FOXO3 affects the expression of NK1R, we overexpressed exogenous NK1R alone in vitro. The results showed that OVX induced a decrease in FOXO3 and NK1R levels, and FOXO3 overexpression could significantly improve the expression of NK1R. The same results were obtained for both protein and mRNA relative levels (Figure 5A, 5B). Alizarin red staining suggested that OVX-induced reduced calcification levels were also inhibited by FOXO3 overexpression (Figure 5C). FOXO3 binds to the NK1R promoter region and increases the transcription level of NK1R (Figure 5D).

Discussion

Pain and loss of joint function caused by postmenopausal OA are one of the main causes of motor dysfunction and chronic disability in postmenopausal women(20, 21). The prevalence of OA in postmenopausal women is greatly increased, and the secretion of estrogen is closely related to the severity of OA, suggesting that estrogen is involved in the occurrence and development of postmenopausal OA. Preclinical studies and clinical trials of estrogen therapy for OA have reported inconsistent results. Several experiments have proved the role of ZGW in the prevention and treatment of PMOP diseases, but the specific active components and the potential mechanism of action are poorly understood.

In our lab, a comorbidity model of PMOP and postmenopausal OA rats was established by OVX. It was found that the femur trabecular bone of the model rats was sparse, disorganized and spaced, the number of trabecular bones decreased, the separation degree of trabecular bone increased, and the bone density decreased significantly, indicating that severe PMOP occurred in this animal model. Therefore, in this study, the PMOP disease model was established by the OVX. Rats were fed with positive drugs and ZGW of different doses, and their serum, femur and tibia were collected. We found that the reduced expression of NK1R was related to the transcription factor FOXO3 in the extracted BMSC. In further experiments, it was found that the therapeutic effect of ZGW on PMOP might be due to the increase of FOXO3.

NK1R is expressed by osteoblast osteoclasts and their
progenitors(22). SP and other neuropeptides by binding to receptors to control the function of osteoclasts and osteoblasts and participate in bone repair, reconstruction and growth. The accelerated effect of NK1R-related signaling pathway on bone resorption of osteoclasts has been previously reported. On the other hand, the effect of NK1-related signaling pathways on bone formation is not fully understood. However, in animal models of osteoporosis, many animal studies have demonstrated the role of NK1R-related signaling pathways in bone formation and the pathogenesis of osteoporosis(19, 23). All of the above-mentioned studies are limited in that they only used animal models. Nevertheless, it seems that the pathophysiology of bone-related diseases such as osteoporosis is associated with the SP-NK1R pathway. In our experiment, the changes of NK1R in osteoporosis were first verified in animal models, and the results obtained were consistent with previous studies. In the model of OVX-induced osteoporosis, the NK1R signaling pathway is inhibited. At the same time, the extracted primary cells showed higher lipid differentiation ability and lower osteogenic differentiation ability. Interestingly, we also found that ZGW had a significant patient effect on NK1R reduction. Subsequently, we predicted the transcription factor of NK1R and found that the transcription factor FOXO3 might be its effective transcription factor.

FoxOs represent a subclass of the large family of forkhead proteins characterized by the presence of a wing-helix DNA binding domain called forkhead box. FoxOs are major targets of the insulin-IGF1 signaling pathway, which inhibits FoxOs activity through Akt-mediated phosphorylation(24-26). Mouse models of loss and acquisition of FoxOs function have demonstrated that FoxOs is an important regulator of osteoclast differentiation and bone resorption(27-29). Specifically, the combined loss of FoxO1, 3, and 4 in the myeloid system promotes cell proliferation, osteoclast formation, and bone resorption, resulting in reduced trabecular and cortical bone mass(30). In contrast, FoxO3 overexpression attenuates osteoclast formation and bone resorption and increases bone mass(31). FoxOs family has shown its unique role in the reverse of osteoporosis, and we found that it can be protected by ZGW and act as a transcription factor to increase the transcription of NK1R.

The pathological mechanism of postmenopausal osteoporosis is complex, affecting a variety of growth factors and hormones, and involving many signaling pathways, and our study is just one of them. ZGW was considered to have a good effect on the prevention and treatment of PMOP in the experimental reports. Although our experiment revealed that the mechanism of ZGW in the treatment of osteoporosis may be through the regulation of NK1R and its transcription factor FOXO3, the specific components of ZGW still need to be further explored and studied. ZGW was a compound with complex components and it was difficult to identify the active components. We have identified 11 potential anti-osteoporosis compounds in ZGW by ULTRA-performance liquid chromatography-high resolution mass spectrometry(13). Perhaps we should conduct more in-depth research into the effects and mechanisms of the various ingredients to identify the most effective ones.

In conclusion, the results of this study illustrated that the regulation of FoxO3 and NK1R played a role and contributed to the mechanism of ZGW underlying the increase in the bone mass in the OVX rat model. One or more of the principal components of ZGW may be a potential herbal alternative for PMOP treatment.

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest
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.

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References

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