

Jingui Shenqi Pills Prevents and Treats Postmenopausal Osteoporosis via Regulating the BMP/Smad Signaling Pathway

Qian Zhang

Shandong University of Traditional Chinese Medicine

Xu Yang

Shandong University of Traditional Chinese Medicine

Ke Ma

Shandong University of Traditional Chinese Medicine

Yanan Zhang

Shandong University of Traditional Chinese Medicine

Xu Tian

Shandong University of Traditional Chinese Medicine

Yuan Wang

Shandong University of Traditional Chinese Medicine

Xiaochun Han

Shandong University of Traditional Chinese Medicine

Jin Wang

Shandong University of Traditional Chinese Medicine

Shijun Wang

Shandong University of Traditional Chinese Medicine

Huaxin Wang (✉ whx215@163.com)

Shandong University of Traditional Chinese Medicine <https://orcid.org/0000-0002-8293-4231>

Research

Keywords: Traditional Chinese Medicine, Jingui Shenqi pills, Postmenopausal Osteoporosis, BMP/Smad signalling Path

Posted Date: July 7th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-669680/v1>

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***Jingui Shenqi* pills Prevents and Treats Postmenopausal osteoporosis via Regulating the
BMP/Smad Signaling Pathway**

Qian Zhang^{a,b,1}, Xu Yang^{c,1}, Ke Ma^{a,b}, Yanan Zhang^{a,b}, Xu Tian^{b,c}, Yuan Wang^{a,b}, Xiaochun Han^{a,b},
Jin Wang^c, Shijun Wang^{a,b,*}, Huaxin Wang^{a,b,*}

a. College of Traditional Chinese Medicine, Shandong University of Traditional Chinese Medicine, Jinan, Shandong Province 250355, China

b. Shandong Co-Innovation Center of Classic TCM Formula, Shandong University of Traditional Chinese Medicine, Jinan, Shandong Province 250355, China

c. Shandong University of Traditional Chinese Medicine, Jinan, Shandong Province 250355, China

Running title: *Jingui Shenqi* pills in the Treatment of postmenopausal osteoporosis

Address Correspondence to:

Dr Huaxin Wang, Shandong Co-Innovation Center of Classic TCM Formula, College of Traditional Chinese Medicine, Shandong University of Traditional Chinese Medicine, No 4655, University Road, Changqing District, Jinan, Shandong 250355, PR China, Tel/Fax: +86-531-89628077, Email: whx215@163.com;

Or Dr Shijun Wang, Shandong Co-Innovation Center of Classic TCM Formula, College of Traditional Chinese Medicine, Shandong University of Traditional Chinese Medicine, No 4655, University Road, Changqing District, Jinan, Shandong 250355, PR China, Tel/Fax+86-531-89628077, Email: pathology@163.com.

¹Qian Zhang and Xu Yang contributed equally to this work.

Abstract

Background: Jingui Shenqi (JGSQ for short) pills is a traditional Chinese medicine formula, which has the functions of warming and tonifying kidney-yang, generating essence and filling marrow, warming tendons and veins and bones, and improving bone mineral density. The aim of this study was to investigate the effect and mechanism of JGSQ pills in preventing and treating postmenopausal osteoporosis.

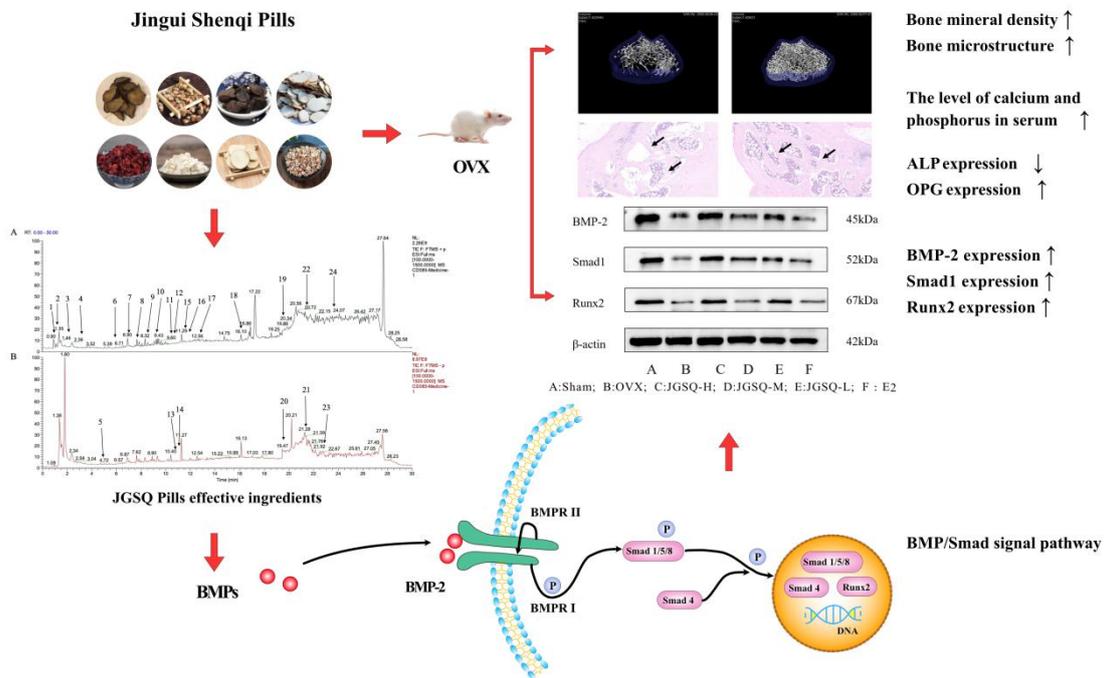
Methods: Twelve-week-old SPF female SD rats (n=48) were used in this study. Following the ovariectomy operation (n=40), the rats were randomly divided into the model group, (high, medium, and low dose groups) treated with Jingui Shenqi pills and estradiol group. Recorded the weight gain of rats and calculated the uterine coefficient; To detect the expression of serum calcium, phosphorus, ALP and OPG; HE staining was used to detect the pathological changes of femur; In all the groups, Micro-CT was used for detection of bone mineral density and bone microstructure; and gene expression of BMP-2, Smad1, and Runx2 in rat bone tissue was determined by RT-PCR and Western Blot methods.

Results: Compared with the sham operation group, rats in the model group had the highest increase in body weight and the lowest uterine coefficient. Each of the treatment groups had a modest increase in weight gain. Micro-CT and HE staining showed a decrease in bone mineral density in the model group with shorter, thinner, broken, and osteoporotic trabeculae of the femur. The bone mineral density in Jingui Shenqi pills treatment groups had a significant increase with an improved bone microstructure and intact bone trabecular as compared to the model group. Serum ALP in the model group was significantly higher than in the sham operation group but the serum calcium, phosphorus and OPG was significantly lower. The Jingui Shenqi pills treatment groups and the estradiol treated group had lower serum ALP and increased serum calcium, phosphorus and OPG. There was decreased gene expression of BMP-2, Smad1, and Runx2 in the bone tissue of the model group compared to the sham operation group. BMP-2, Smad1, and Runx2 gene expression in Jingui Shenqi pills treated group and estradiol treated group was significantly higher than that of the model group.

Conclusion: Jingui Shenqi pills improve the microstructure of bone tissue and increase bone mineral density thus resolving osteoporosis. This is achieved by regulating BMP/Smad signaling pathway and increasing the expression of osteogenic factors BMP-2, Smad1, and Runx2.

Keywords Traditional Chinese Medicine; Jingui Shenqi pills; Postmenopausal Osteoporosis; BMP/Smad signalling Pathway.

Graphical abstract:



1. Introduction:

Osteoporosis is a metabolic disease that frequently occurs in elderly and postmenopausal women. It is mainly characterized by damage of the bone tissue microstructure, decrease of bone mineral density, and increased risk of fractures [1] [2]. Environmental and genetic factors accelerate the occurrence and development of osteoporosis, of which the imbalance of bone remodeling caused by reduced bone formation is a key factor in the occurrence of osteoporosis[3]. Statistics show that worldwide, 1,000 people are fractured due to OP every hour, and there are about 900 million fractures each year[4], of which the incidence of postmenopausal osteoporosis is about 30% in people over 50 years of age[5], and the resulting fractures are the main cause of middle-aged and elderly visits, which seriously affect the physical and mental health of middle-aged and elderly people, bring a huge burden to families and society, and become an important public health problem currently faced .

Modern medicine believes that the dynamic balance of osteoblasts and osteoclasts is the key to maintain bone remodeling, thereby maintaining the balance of bone metabolism[6]. Bone remodeling is regulated by various factors, such as estrogen levels, intestinal bacteria, oxidative stress, and inflammatory responses[7]. At present, the commonly used anti-osteoporosis drugs in clinical practice include bisphosphonates, parathyroid hormone and hormone replacement therapy. However, while exerting the role of targeted therapy, the above drugs ignore the integrity of the body. Long-term or high-dose administration will increase the risk of cardiovascular disease, cervical cancer, kidney stones and gastrointestinal diseases[8]. Therefore, it is important to continue exploring for safe and effective treatment.

Traditional Chinese medicine seeks the root cause of the disease, pays attention to the occurrence, development, and disease changes to bring a balance to yin and yang. Traditional Chinese medicine has the advantage of minimal side effects and high efficacy in the treatment of postmenopausal osteoporosis. The multiple compounds in a traditional Chinese medicine preparation act on multi-targets and multi-pathways to promote bone formation. Traditional Chinese medicine also relieves the bone pain associated with osteoporosis[9]. Traditional Chinese medicine theories, postulate that the kidney controls bone marrow generation by regulating bone metabolism. Jingui Shenqi pills (JGSQ for short) are a classic prescription for warming and act

as a tonic for kidney yang which was first published in the synopsis of the Golden Chamber. It is composed of dried *Rehmannia glutinosa*, yam, *Cornus officinalis*, Poria, alisma, moutan, aconite, and cassia twig. Currently, it is clinically used in the treatment of kidney yang deficiency[10] [11] [12].

Research has shown Jingui Shenqi pills relieve osteoporosis-associated bone pain[13]. Additionally, long-term treatment with Jingui Shenqi pills in postmenopausal osteoporosis resulted in a similar increase in bone mineral density as alendronate[14]. Jingui Shenqi pills were also shown to increase estrogen in *in vivo* studies carried out in ovariectomized rats. Jingui Shenqi pills also improved osteoblast vitality, ALP activity, and Collagen I protein expression[15]. Jingui Shenqi pills were also shown to promote bone differentiation in an *in vitro* study, by promoting the expression of ALP, RUNX2, and OCN[16]. However, the mechanism of Jingui Shenqi pills in preventing and treating PMOP has not been fully elucidated. Current studies have shown that the mechanism of traditional Chinese medicine in preventing and treating postmenopausal osteoporosis is related to the BMP/Smad signaling pathway, which can regulate all processes of osteoblast proliferation, differentiation, and mineralization, and its abnormal function can cause decreased bone mass and reduced bone formation, leading to osteoporosis[17] [18]. The BMP/Smad signaling pathway can regulate downstream osteoblast transcription factors, promote bone formation, and increase bone mineral density[19] [20] [21].

Therefore, in this experiment, ovariectomy was used to duplicate the postmenopausal osteoporosis model to observe the effect of Jinkui Shenqi Pills on calcium, phosphorus and bone formation markers in the serum of OVX rats; HE staining was used to observe the pathological changes of the femur; micro-CT was used to detect the bone mineral density and bone microarchitecture of rats; qRT-PCR and Western blot were used to detect the gene expression of BMP-2, Smad1 and Runx2 in the femur of rats to further elucidate the mechanism by which Jinkui Shenqi Pills improved osteoporosis in OVX rats. It further enriches the theory of "kidney main bone" and provides theoretical basis and scientific basis for the prevention and treatment of osteoporosis by traditional Chinese medicine.

2. Materials and methods

2.1 JGSQ pills preparation

All the traditional Chinese medicines of JGSQ were purchased from Shandong Xinzhonglu Hospital (Jinan, China). The preparation of JGSQ is composed of eight herbal medicines as follows: *Rehmannia glutinosa* 24g, *Dioscorea zingiberensis* 12g, *Cornus officinalis* 12g, *Poria* 9g, *Alisma alisma* 9g, moutan bark 9g, aconite 3g, and cassia twig 3g. The herbal materials were decocted in eight volumes of distilled water for 1 hour. A filtrate was then obtained by double filtration. The filtrate was then concentrated to a high dose of 1.7g/ml (twice the clinical dose), a medium dose of 0.85g/ml (clinically used dose), and a low dose of 0.425g/ml (half the clinical dose). Medium dose is the original amount of classical prescription.

2.2 Qualitative analysis for ingredients in JGSQ standard decoction

To a plant decoction filtrate of 200 μ l, 1mL of methanol: water (8:2 V:V) was added. The solution was mixed by vortexing and centrifugation was done at 4 °C (20000xg, 10 minutes), The supernatant was filtered with a 0.22 μ m membrane, and the filtrate was analyzed by HPLC. Ion source: electrospray ionization source (ESI); Scanning mode: positive and negative ion switching scanning; Detection method: Full mass/dd-MS2; Resolution: 70000(full mass), 17500(dd-MS2); scanning range: 100.0-1500.0m/z, Spray Voltage:3.8kV(Positive), Capillary Temperature:300°C. An RP-C18 column (150 \times 2.1mm 1.8 μ m, Welch) was used for all analyses and maintained at 30 °C at a flow rate of 0.3 ml/min. , The mobile phase comprised 0.1% formic acid (v/v) (A) and acetonitrile (B) (Table 1).

2.3 Animals model establishment and drug administration

12 week-old female Sprague Dawley rats (n=48) were obtained from the Laboratory Animal Center of Shandong University of Traditional Chinese Medicine (Shandong China). They were maintained in a 12 h light-dark cycle, at a controlled temperature (22-24°C) and humidity (50-60%). Standard laboratory rodent diet and water were provided ad libitum. The rats were subjected to adaptive feeding for three days before the experiment. Complete bilateral ovariectomy was then carried out in all rats (n=40) except the rats allocated to the sham group (a sham operation involving removal of the same amount of fat near the ovary was carried out). Five days after the operation, the rats were allocated to six groups of 8 rats as follows: sham (sham-operated rats + saline), OVX (OVX rats + saline), high-dose JGSQ pills (OVX + JGSQ-H, 1.7 g/kg/d), medium-dose JGSQ pills(OVX + JGSQ-M, 0.85 g/kg/d), Low-dose JKSQ pills(OVX + JGSQ-L, 0.425 g/kg/d), estradiol (OVX + E₂, 0.1 g/kg body weight).The medium dose of JGSQ and Estradiol was equivalent to clinically used doses.This experiment was reviewed by the Ethical

Review Committee of Experimental Animal Welfare of Shandong University of traditional Chinese Medicine, and met the welfare and ethical requirements of experimental animals and animals (Batch number: SDUTCM2018112901).

2.4 Serological index detection

Analysis of calcium and phosphorus (C004-2, C006-1-1, Nanjing Jiancheng) in serum was done using an automatic biochemical analyzer (AU2700, Beckman Technology Co., Ltd.). Serum markers of bone formation: ALP (A059-2, Nanjing Jiancheng) and OPG(E-EL-R0050c, Wuhan Elabscience) levels in the serum were determined using ELISA kits per the manufacturer's instructions. The bone turnover markers were assessed at 450 nm using an enzyme-linked immune detector (American Boteng instrument Co., Ltd. VT 05404-0998).

2.5 Hematoxylin and eosin (H&E) staining

The left femur bones were collected, fixed in 10% neutral formaldehyde for 72 h, then removed and decalcified in 10% ethylenediaminetetraacetic acid solution (pH 7.4) at 4°C for 4 weeks. Paraffin was then used to embed the fixed samples. The samples were then cut into 4 μm slices which were stained with H&E stain and observed under a light microscope.

2.6 BMD and Micro-CT examination

Local bone mineral density (g/cm^2) and trabecular microstructure (BV/TV, BS/BV, Tb.Th, Tb.N, SMI, Tb.Sp and BMD) of the right distal femur were measured and analyzed by Bruker-Micro CT (NEMO, Pingsheng Medical Technology Co., Ltd.). Following the CT scan, the cortical and trabecular bones of the femur were analyzed by Skyscan NRecon software. The 3D and 2D sections of femoral cortical and trabecular bone were reconstructed.

2.7 qRT-PCR

Total RNA was extracted from the bone tissue with Vazyme R401-01RNA isolator per the manufacturer's instructions. Reverse transcription was then carried out using Vazyme R323-01kit. Primer sequences were designed based on GenBank sequences with Primer Premier 5.0 software

(Table 2) by Shanghai Shenggong Co., Ltd., China. ACTB was used as an internal control. SYBRGreen PCR kit (Vazyme Q711-02/03) was used to prepare the reaction master mix, Fluorescencequantitative PCR (Bio-Rad, USA) was used for mRNA amplification and quantification. The reaction conditions were: denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5-10 s, annealing at 60 °C for 30 s, and separation at 95°C for 15 s, 60°C for 60 s and 95°C for 15 s. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method.

2.8 Western blot

The total protein was extracted from bone tissue using RIPA lysis buffer (P0013B, Beyotime) after pulverizing in liquid nitrogen. The concentration of the protein was determined using a BCA protein kit (G2026, Servicebio). The protein was then mixed with loading buffer and boiled at 100 °C for 5 min to denature it. 30µg of total protein was separated by 10% SDS-PAGE and transferred into 0.45 µm polyvinylidene fluoride (A29280264, GE Healthcarelifescience) membranes. After blocking with 5% nonfat dried milk for one and a half hours, membranes were incubated with primary antibodies at room temperature: rabbit anti-BMP-2 (Ab14933, Abcom), rabbit anti-Smad1 (10429-1-AP, Proteintech), and rabbit anti-Runx2 (Bs-1134R, Bioss) respectively for 2 hours. The membranes were then incubated with goat anti-rabbit IgG (LY192274, Servicebio) as the secondary antibody for 1 h at room temperature. The blots were developed with ECL reagent (B500023, Proteintech). The total protein was compared with β-actin as an internal reference, and the gray values of internal reference and target bands were quantified by Image-ProPlus6.0 software.

2.9 Statistical analysis

SPSS 22.0 (SPSS Inc., Chicago, IL, USA) was used to perform the statistical analyses and the data were expressed as mean ± SEM. Differences between two groups were compared by *t*-test, while the comparison between three or more groups were performed through one-way analysis of variance (ANOVA) followed by Dennett's test in order to detect intergroup differences. $P < 0.05$ was considered as statistically significant.

3. Results

3.1 Identified compounds in JGSQ pills

The components of the JGSQ (Salsolinol, choline, uracil, gallic acid, caffeic acid, et al) filtrate was verified by Q-Orbitrap high-resolution liquid chromatography-mass spectrometry under optimized conditions. The main chemical components in JGSQ freeze-dried powder were detected by comparing with the retention time and UV spectrum of the reference standard. A total of 24 peak signals were obtained. The chemical components were also identified (Table 3 and Fig 1).

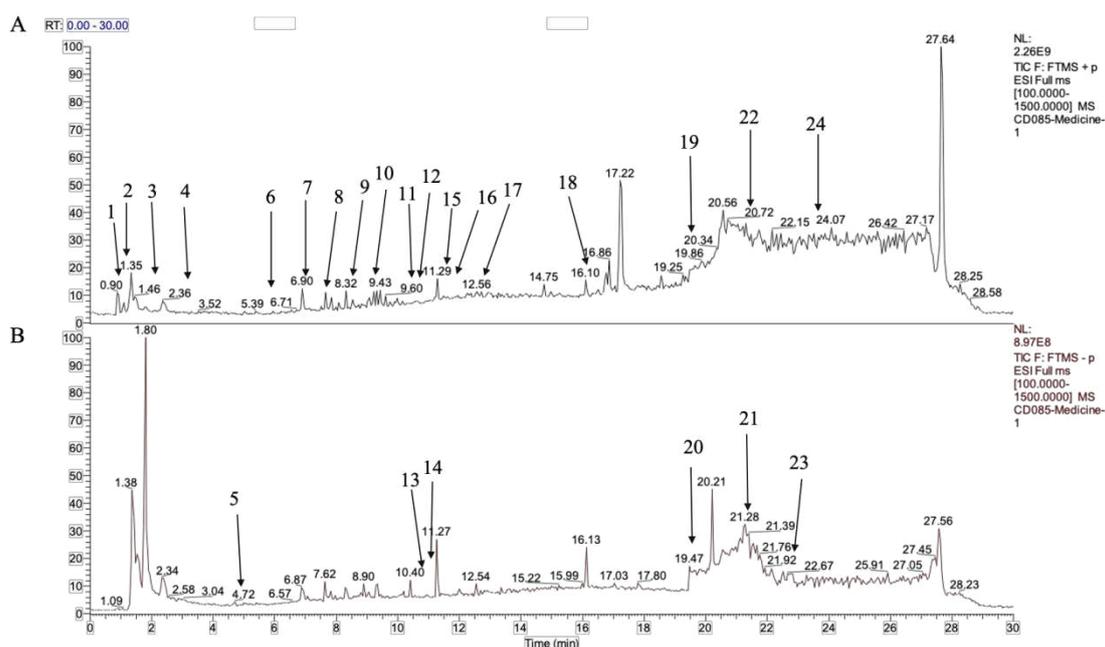


Figure.1 Total ion flow patterns of JGSQ pills in positive (A) and negative ion (B) modes.

3.2 The general condition of rats

Rats in the Sham group, had soft, shiny hair, were sensitive to activity. Rats in the OVX group had a significant decrease in activity as compared to the other treatment groups. The body weight of the rats was recorded weekly during the 3 months. Compared with the Sham group, the OVX group had the highest weight gain and lowest uterine coefficient, while JGSQ-H, JGSQ-M, JGSQ-L, and E2 groups recorded a lower weight gain (Fig.2A-2B), and an insignificant change in the uterine coefficient (Fig.2C).

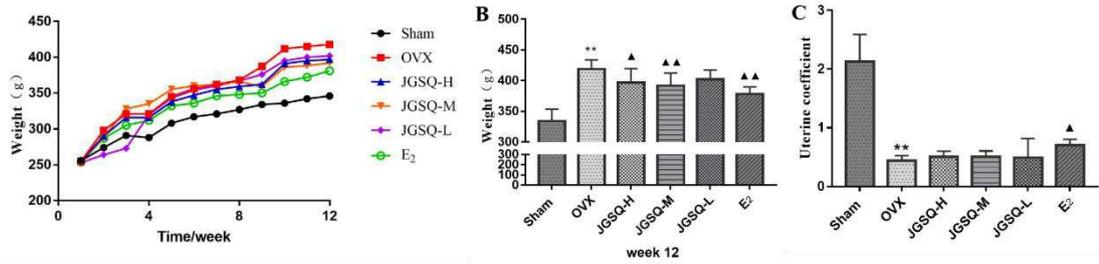


Figure.2 The general condition of rats. A. Effect of Jingui Shenqi pills on rats body weight. B. Body weight of rats in each group at Week 12. C. Effect of Jingui Shenqi pills on uterine coefficient different rat groups. *represents a comparison with the sham group, $*P < 0.05$, $**P < 0.01$; ▲represents a comparison with OVX group, $\blacktriangle P < 0.05$, $\blacktriangle\blacktriangle P < 0.01$.

3.3 Biochemical index

After 3 months of treatment, the serum levels of calcium and phosphorus in the OVX group were significantly lower than in the Sham group. However, the serum levels in the JGSQ-H group were significantly higher than in the OVX group. There was no significant difference between the treatment groups (Fig.3A-3B).

3.4 Serum bone formation markers

Compared with the Sham group, the serum ALP levels in the OVX group were significantly higher, and the serum OPG was significantly lower. The JGSQ-H, JGSQ-M, JGSQ-L, and E₂ treated groups had a decrease in the levels of ALP and an increase in serum OPG (Fig.3C-3D).

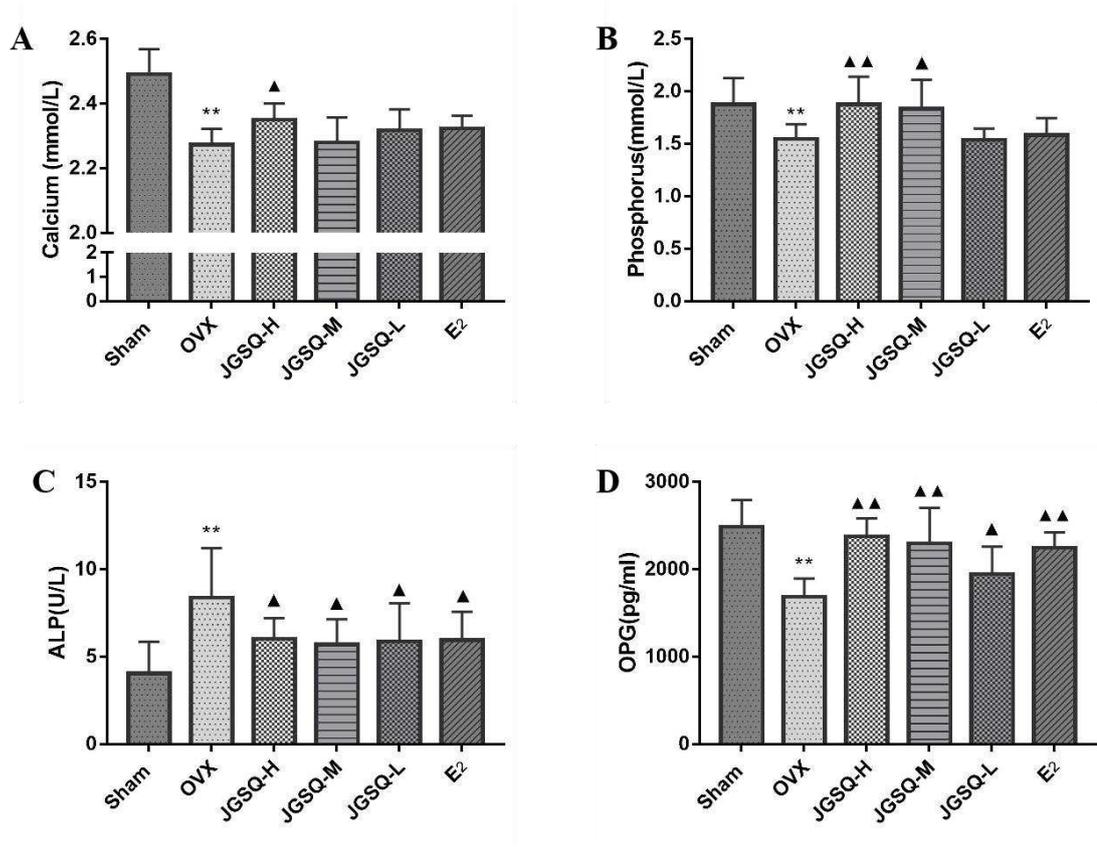


Figure.3 Effect of Jingui Shenqi Pills on Serum Parameters in Ovariectomized Rats. A,B. Effect of Jingui Shenqi pills on calcium and phosphorus in serum of ovariectomized rats. C,D. Effect of Jingui Shenqi pills on serum ALP and OPG in ovariectomized rats. *represents a comparison with the sham group, $*P < 0.05$, $**P < 0.01$; ▲represents a comparison with OVX group, $\blacktriangle P < 0.05$, $\blacktriangle\blacktriangle P < 0.01$.

3.5 Histopathological analysis

The results of HE staining showed that the bone tissue structure in the Sham group was stable, the trabecular was dense and tidy, and the cortical and cancellous bones could be seen. In the OVX group, the bone tissue structure was seriously damaged, with a reduced trabecular volume and thickness, and a damaged medullary cavity space. Treatment with Jingui Shenqi pills showed an improvement in the bone microstructure, increased bone trabeculae, and an increase in the bone connection (Fig.4).

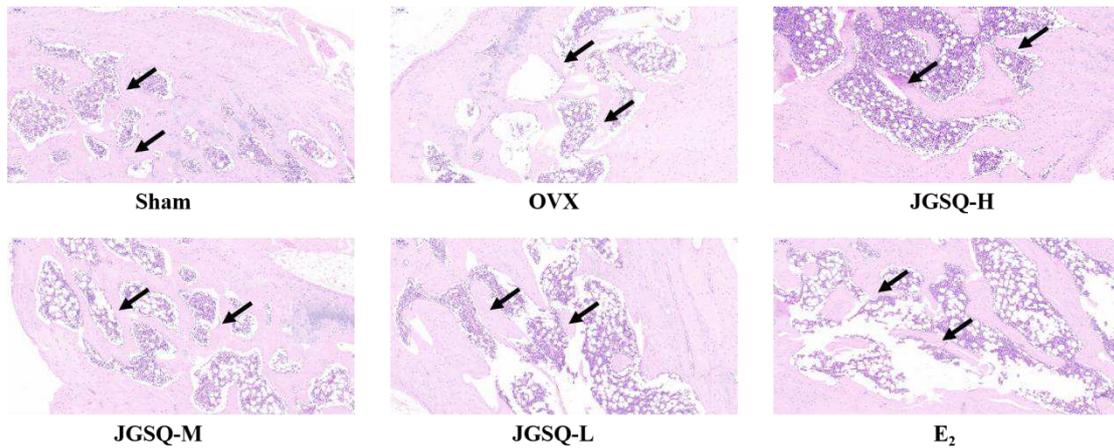


Figure.4 Effect of Jingui Shenqi Pills on Pathological Changes of Femur in Ovariectomized Rats. Representative photographs of H&E stained femoral sections from each group ($\times 100$). Arrow (black): bone.

3.6 BMD and bone microstructure analysis

The results of the previous preliminary experiment showed that the bone mineral density of the JGSQ-L group was not significantly improved compared with the model group, so only the JGSQ-H and JGSQ-M groups were tested. Compared with the Sham group, BMD decreased significantly in the OVX group but had a significant increase in the JGSQ-M group. Three-dimensional imaging of the femur of rats in the Sham group showed the trabecular as being moderate in size, thickness, compact arrangement, and had a good bone connection, while the OVX group, had shorter, thinner, fewer, sparse, and fractured trabecular, with loose shape. After treatment with Jingui Shenqi pills, the bone microstructure improved significantly and had an intact trabecular structure (Fig.5).

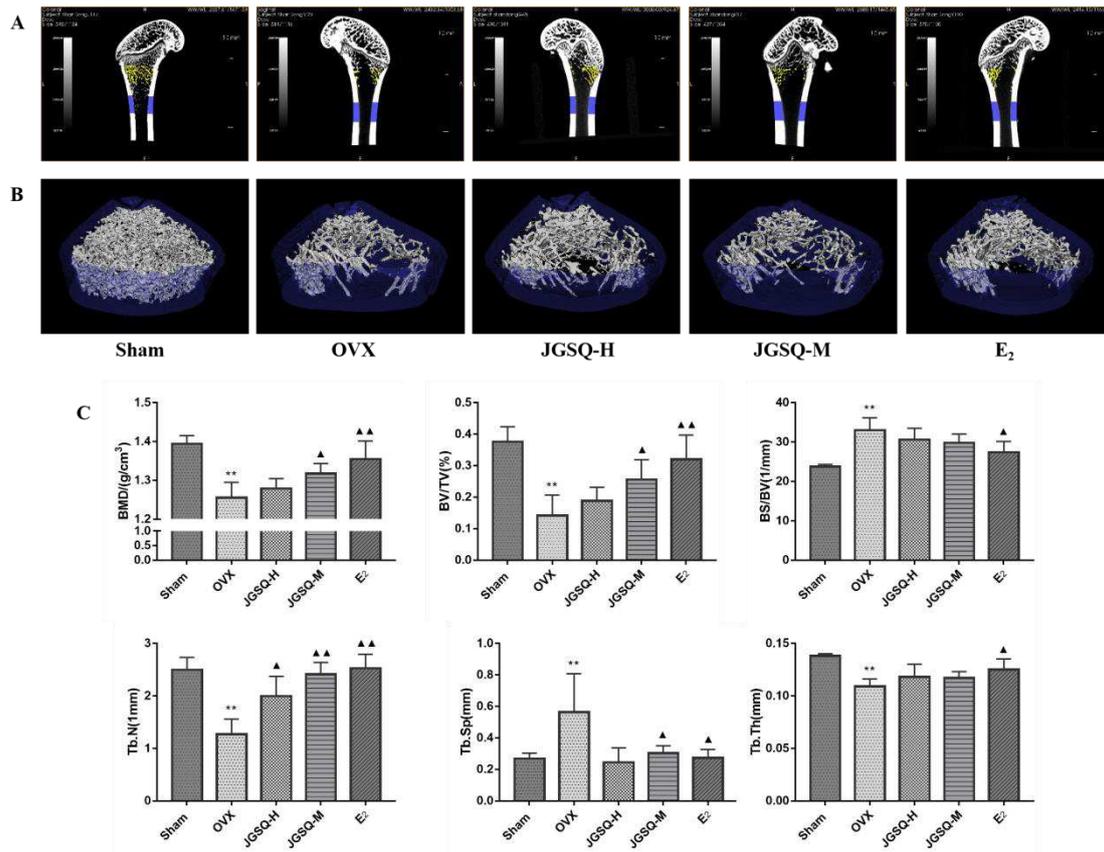


Figure.5 Effect of Jingui Shenqi Pills on Bone Mineral Density and Microstructure in Ovariectomized Rats. A. Representative 2D images of the vertical plane of the bone microstructure of the right distal femur (n = 3). B. Effects of JGSQ pills on the structural thickness of the right distal femur as displayed on 3D models. C. Effect of Jingui Shenqi pills on bone mineral density in ovariectomized rats, including BMD (bone density) BV/TV (Bone Volume/Total Volume), BS/BV (Bone Surface/Bone Volume), Tb.N (trabecular number), Tb.Sp (trabecular separation) and Tb.Th (trabecular thickness). *represents a comparison with the sham group, $P < 0.05$, $P < 0.01$; ▲ represents a comparison with OVX group, $P < 0.05$, $P < 0.01$.

3.7 Expression of mRNA in bone tissue

The expression of BMP-2, Smad1, and Runx2 mRNA in bone tissue was significantly decreased in the OVX group than the Sham group. However, the expression of BMP-2, Smad1 and Runx2 mRNA in bone tissue in the Jingui Shenqi pills treated group and E2 treated group was higher than in the OVX group (Fig.6A).

3.8 Protein expression in bone tissue

Western Blot analysis showed that the expression of BMP-2, Smad1, and Runx2 protein in bone tissue in the OVX group was significantly lower than in the Sham group, while the expression of BMP-2, Smad1, and Runx2 protein in bone tissue in Jingui Shenqi pills treated group and E2 treated group was higher than in the OVX group ($P < 0.05$). There was no significant difference between the treatment groups (Fig.6B-6C).

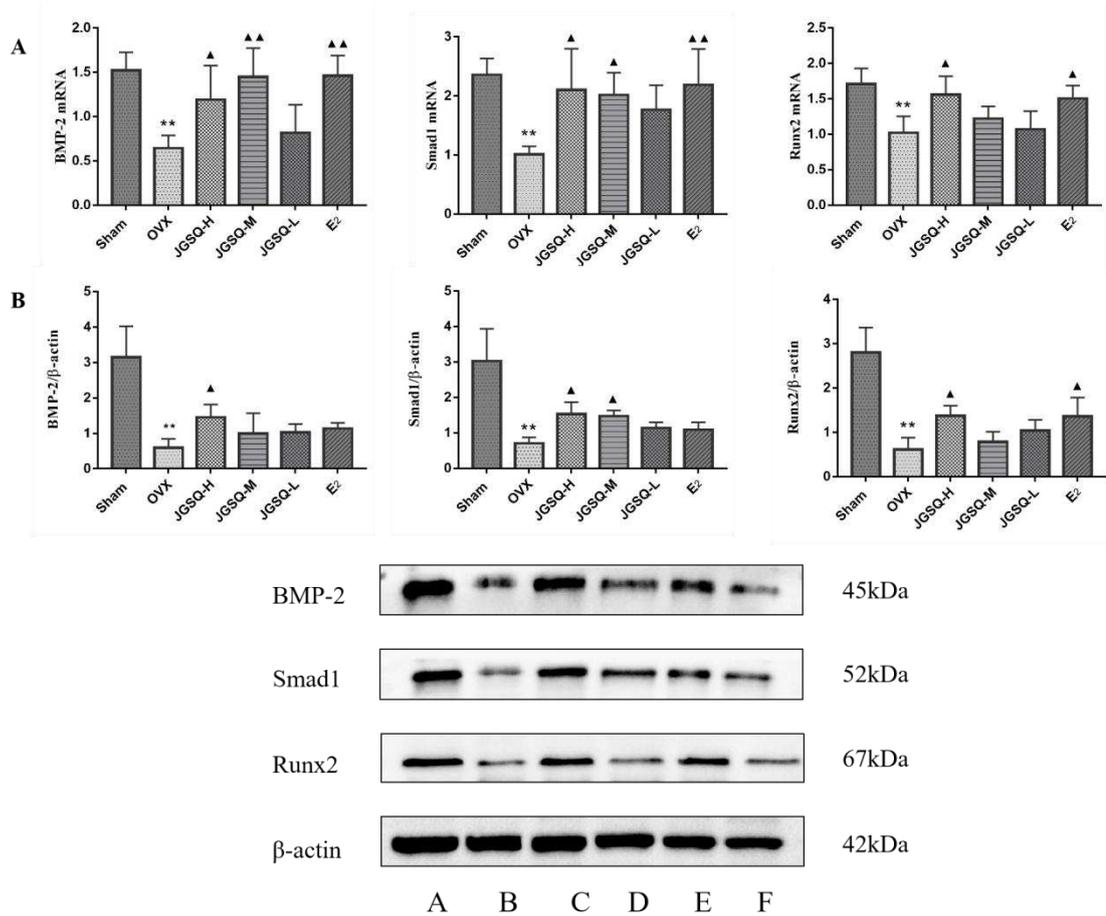


Figure.6 Effect of Jingui Shenqi Pills on BMP-2, Smad1, Runx2 gene expression in bone tissue of ovariectomized rats. A. The mRNA expression of BMP-2, Smad1 and Runx2 in rat bone tissues was measured by qRT-PCR. B. Western blot was used to detect the protein expression of BMP-2, Smad1 and Runx2 in rat bone tissues. C. Representative WB photographs of BMP-2, Smad1 and Runx2 were viewed, and β -actin was used for normalization.*represents a comparison with the sham group, $*P < 0.05$, $**P < 0.01$; ▲represents a comparison with OVX group, $\blacktriangle P < 0.05$, $\blacktriangle\blacktriangle P < 0.01$.

4. Discussion

The majority of the middle-aged and elderly people have a Yang deficiency. They often experience symptoms such as sore waist and knees, fear of cold, tinnitus, and diarrhea. Kidney

yang is the foundation of the whole body yang. A deficiency in kidney yang results in bones lacking normal qi and blood nourishment, failure of joints to flex and extend normally, and impaired support resulting in osteoporosis. Jingui Shenqi pills prescription is derived from the synopsis of the Golden Chamber and is known as "the first prescription tonic for kidney yang through the ages". Jingui Shenqi pills can invigorate the kidney yang, gasify and return to normal, replenish the kidney yang, smooth the blood flow, and nourish the muscles, veins, and bones[22]. Qi Jicong found that Jingui Shenqi pills can relieve the pain caused by hyperosteogeny of the calcaneus and effectively treat "bone impotence"[23]. Jingui Shenqi pills were also reported to increase bone mineral density[24]. Fructus Corni Neoglycoside I has also been shown to promote the proliferation and differentiation of osteoblasts[25], and significantly increase the mRNA expression levels of Wnt2, BMP2, β -catenin, OPG, and NOX4 in osteoblasts[26]. Additionally, dried *Rehmannia glutinosa* has been shown to significantly inhibit the effects of OVX on the femur and lumbar vertebrae in rats[27].

The decline of ovarian function in menopause and the sudden drop of estrogen levels leads to a fat metabolism disorder, hyper appetite, and decreased activity, which reduces the basal metabolic rate and increases the fat content resulting in obesity[28] [29]. Excessive differentiation of adipocytes reduces osteoblast differentiation and bone formation, resulting in reduced bone formation and increased bone resorption, thus a decrease in bone mineral content, and osteoporosis[30] [31]. This study revealed that Jingui Shenqi pills reduce the weight gain induced by ovariectomy, increases the trabecular volume, and thickness, reduces trabecular separation, increases bone mineral density, and prevent the occurrence of postmenopausal osteoporosis.

Changes in the levels of calcium and phosphorus are important indicators that reflect on bone metabolism and changes to the whole body[32] [33] [34]. ALP (an intracellular enzyme in osteoblasts), is an important marker of bone formation[35]. When bone metabolism is exuberant, osteoblasts are active, and the ALP activity is increased. Estrogen secretion is decreased in OVX rats, resulting in a high bone metabolic state, that is, bone formation is less than bone resorption, simulating the bone metabolism in postmenopausal women[36]. OPG is secreted by osteoblasts and vascular endothelial cells and inhibits the differentiation and maturation of osteoclast precursors. Therefore, the level of serum OPG plays an important role in the early diagnosis of

osteoporosis[37] [38]. Jingui Shenqi pills regulate bone metabolism and balance bone remodeling by increasing the levels of serum calcium, phosphorus, and OPG, and reducing the level of serum ALP.

Bone morphogenetic protein (BMP) is a family of proteins with multiple biological functions, which binds receptors on the target cell membrane. This results in activation of the BMP/Smad signaling transduction pathway[39]. The BMP-2 ligand secreted by osteocytes binds to the receptors BMPRI and BMPRII on the cell membrane forming a complex. The phosphorylated type II receptor activates the type I receptor, which then activates and phosphorylates receptor-regulated Smads protein (Smad1/5/8) The regulated smads then bind to Smad4 and translocates to the nucleus. The complex binds to DNA binding protein and regulates the expression of downstream transcription factors Runx2 and OSX, thus regulating the transcription of genes related to different stages of osteogenic differentiation[16].

BMP/Smad signaling pathway is a classical pathway in promoting osteoblast proliferation and differentiation in bone metabolism[40]. BMPs are an important osteogenic factor in inducing BMSCs to differentiate into osteoblasts and may result in increased osteoblast ALP activity, collagen synthesis, nerve cell differentiation, and kidney development[41] [42]. Smad protein mainly regulates bone metabolism through TGF- β / BMP pathway, which directly or indirectly induces the proliferation, differentiation, and apoptosis of osteoblasts and osteoclasts[43] [44]. BMP/Smad signal pathway wholly regulates osteogenic differentiation, thus regulating osteogenic metabolism[45]. The study findings revealed a decrease in mRNA and protein levels of BMP-2, Smad1, and Runx2 in the OVX group compared to the sham group. Treatment with a high, medium, and low dose of Jingui Shenqi pills, increased mRNA and protein levels of BMP-2, Smad1, and Runx2. This proves that Jingui Shenqi pills are effective in preventing and treating PMOP by activation of the BMP/Smad signaling pathway. Jingui Shenqi pills also promote bone formation and improve osteoporosis via regulation of the BMP/Smad signaling pathway and increasing the expression of BMP-2, Smad1, and Runx2.

5 Conclusions

This study revealed that JGSQ promote bone formation, improves bone tissue microstructure, and increases bone mineral density by regulating the expression of the BMP/Smad signaling pathway. JGSQ offer clinical value in resolving osteoporosis. As a traditional Chinese medicine compound preparation with high safety and efficacy, JGSQ can be used as a preventive and therapeutic drug for osteoporosis for in-depth mining and development.

Abbreviation:

JGSQ-H, high-dose JGSQ pills; JGSQ-M, medium-dose JGSQ pills; JGSQ-L, Low-dose JGSQ pills; E₂, Estradiol; ELISA, enzyme linked immunosorbent assay; qRT-PCR, quantitative RT-PCR; ALP, Alkaline Phosphatase; OPG, Osteoprotegerin; BMP-2, Bone Morphogenetic Protein 2.

Ethics approval and consent to participate

This experiment was reviewed by the Ethical Review Committee of Experimental Animal Welfare of Shandong University of traditional Chinese Medicine, and met the welfare and ethical requirements of experimental animals and animals (Batch number: SDUTCM2018112901).

Consent for publication

Not applicable.

Availability of data and materials

The data used to support the results of this study can be obtained from the first author upon reasonable request.

Competing interests

The authors declare no conflict of interest.

Funding

This project was supported by the Youth Science Foundation of the National Natural Science Foundation of China (82004087), the Science and Technology Innovation team of Shandong

University of Traditional Chinese Medicine (2020-54-19), and the Science and Technology Development Plan of Shandong Province of traditional Chinese Medicine (2017-004).

Authors' contribution

Huaxin Wang is fully responsible for experimental design, paper drafting and finalization. Qian Zhang and Xu Yang contributed equally to this manuscript. Qian Zhang, Xu Yang, Jin Wang, and Xu Tian completed the experiment and carried out statistical analysis. Ke Ma, Yanan Zhang, Yuan Wang and Xiaochun Han put forward constructive suggestions for the article, and Shijun Wang put forward important suggestions for the experimental process and the paper. All authors have read and approved the final version of the manuscript.

Acknowledgements

We are grateful the support from the Shandong Co-Innovation Center of Classic TCM Formula.

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Figure Legends

Figure.1 Total ion flow patterns of JGSQ pills in positive (A) and negative ion (B) modes.

Figure.2 The general condition of rats. A. Effect of Jingui Shenqi pills on rats body weight. B. Body weight of rats in each group at Week 12. C. Effect of Jingui Shenqi pills on uterine coefficient different rat groups. *represents a comparison with the sham group, $*P < 0.05$, $**P < 0.01$; \blacktriangle represents a comparison with OVX group, $\blacktriangle P < 0.05$, $\blacktriangle\blacktriangle P < 0.01$.

Figure.3 Effect of Jingui Shenqi Pills on Serum Parameters in Ovariectomized Rats. A,B. Effect of Jingui Shenqi pills on calcium and phosphorus in serum of ovariectomized rats. C,D. Effect of Jingui Shenqi pills on serum ALP and OPG in ovariectomized rats. *represents a comparison with the sham group, $*P < 0.05$, $**P < 0.01$; \blacktriangle represents a comparison with OVX group, $\blacktriangle P < 0.05$, $\blacktriangle\blacktriangle P < 0.01$.

Figure.4 Effect of Jingui Shenqi Pills on Pathological Changes of Femur in Ovariectomized Rats. Representative photographs of H&E stained femoral sections from each group ($\times 100$). Arrow (black): bone.

Figure.5 Effect of Jingui Shenqi Pills on Bone Mineral Density and Microstructure in Ovariectomized Rats. A. Representative 2D images of the vertical plane of the bone microstructure of the right distal femur ($n = 3$). B. Effects of JGSQ pills on the structural thickness of the right distal femur as displayed on 3D models. C. Effect of Jingui Shenqi pills on bone mineral density in ovariectomized rats. including BMD (bone density) BV/TV (Bone Volume/Total Volume), BS/BV (Bone Surface/Bone Volume), Tb.N (trabecular number), Tb.Sp (trabecular separation) and Tb.Th (trabecular thickness). *represents a comparison with the sham group, $*P < 0.05$, $**P < 0.01$; \blacktriangle represents a comparison with OVX group, $\blacktriangle P < 0.05$, $\blacktriangle\blacktriangle P < 0.01$.

Figure.6 Effect of Jingui Shenqi Pills on BMP-2, Smad1, Runx2 gene expression in bone tissue of ovariectomized rats. A. The mRNA expression of BMP-2, Smad1 and Runx2 in rat bone tissues was measured by qRT-PCR. B. Western blot was used to detect the protein expression of BMP-2, Smad1 and Runx2 in rat bone tissues. C. Representative WB photographs of BMP-2, Smad1 and Runx2 were viewed, and β -actin was used for normalization. *represents a comparison with the sham group, $*P < 0.05$, $**P < 0.01$; \blacktriangle represents a comparison with OVX group, $\blacktriangle P < 0.05$, $\blacktriangle\blacktriangle P < 0.01$.

Figure.7 Graphical abstract: Jingui Shenqi pills Prevents and Treats Postmenopausal osteoporosis via Regulating the BMP/Smad Signaling Pathway.

Table 1 HPLC gradient elution process

Time (min)	Mobile phase A	Mobile phase B
0	98%	2%
0-5	98-80%	2-20%
5-10	80-50%	20-50%
10-15	50-20%	50-80%
15-20	20-5%	80-95%
20-25	5%	95%
25-30	5-98%	95-2%

Table 2: The primer sequences

Primer		Sequence of primers
BMP-2	Forward	5'-TGCGGTCTCCTAAAGGTCG-3'
	Reverse	5'-ACTCAAACCTCGCTGAGGACG-3'
Smad1	Forward	5'-TCAATAGAGGAGATGTTCAAGCAGT-3'
	Reverse	5'-AAACCATCCACCAACACGCT-3'
Runx2	Forward	5'-GCCGGGAATGATGAGAACTA-3'
	Reverse	5'-GGTGAAACTCTTGCCTCGTC-3'
β-actin	Forward	5'-TGTCACCAACTGGGACGATA-3'
	Reverse	5'-GGGGTGTTGAAGGTCTCAAA-3'

Table 3. Main components in JGSQ pills

No.	Assigned identity	Molecular formula	Molecular weight	TR (min)	Area (Max.)
1	Salsolinol	C ₁₀ H ₁₃ NO ₂	179.09433	1.876	146442.6449
2	L-Serin	C ₃ H ₇ NO ₃	105.04288	1.252	140976.2545
3	choline	C ₅ H ₁₃ NO	103.09997	1.114	573034.5195
4	uracil	C ₄ H ₄ N ₂ O ₂	112.02746	2.876	54295.12559
5	gallic acid	C ₇ H ₆ O ₅	170.02036	4.759	679165.5473
6	mussaenosidic acid	C ₁₆ H ₂₄ O ₁₀	376.13643	6.271	49487.24167
7	caffeic acid	C ₉ H ₈ O ₄	180.04187	7.01	261110.5383
8	Loganin	C ₁₇ H ₂₆ O ₁₀	390.15153	7.646	319772.86
9	1,2,3,6-tetra-O-galloyl-β-D-glucose	C ₃₄ H ₂₈ O ₂₂	788.10678	8.579	342536.3267
10	Myristicin	C ₁₁ H ₁₂ O ₃	192.07852	9.33	129656.2855
11	trans-cinnamic acid	C ₉ H ₈ O ₂	148.0512	10.573	63330.4939
12	coumarin	C ₉ H ₆ O ₂	146.03653	10.624	499750.233
13	abscisic acid	C ₁₅ H ₂₀ O ₄	264.13596	10.779	65729.18049
14	quercetin	C ₁₅ H ₁₀ O ₇	302.04227	11.018	369557.0775
15	(-)-Caryophyllene oxide	C ₁₅ H ₂₄ O	220.18231	11.518	141614.446
16	methyl palmitate	C ₁₇ H ₃₄ O ₂	287.28184	11.572	907368.2794
17	3-[2-(3-hydroxyphenyl)ethyl]-5-methoxyphenol	C ₁₅ H ₁₆ O ₃	244.10948	12.956	398562.2315
18	diosgenin	C ₂₇ H ₄₂ O ₃	396.30184	16.115	86761.9485
19	oleanolic acid	C ₃₀ H ₄₈ O ₃	438.34867	19.55	457465.9619
20	ursolic acid	C ₃₀ H ₄₈ O ₃	456.35977	19.572	251869.2106
21	oleic acid	C ₁₈ H ₃₄ O ₂	282.25569	21.301	449661.2603
22	Stearamide	C ₁₈ H ₃₇ NO	283.28693	21.43	176398.883
23	stearic acid	C ₁₈ H ₃₆ O ₂	284.27136	22.845	148504.1647
24	Erucamide	C ₂₂ H ₄₃ NO	337.33348	23.63	1440974.297