

Original Research Article

Shenggu'an exerts anti-osteoporotic effect in rats via TGF β 1-Smad2/3 signal pathway, and enhancement of bone and cartilage metabolism

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Abstract

Purpose: To study the anti-osteoporotic effect of Shenggu'an in rats, and elucidate the mechanism of action involved.

Methods: Forty healthy female SPF mice were randomly divided into control group, saline-treated group, TGF β R II receptor inhibitor group, and shenggu'an group. The expressions of type II collagen (Co1-II) and platelet endothelial cell adhesion factor (CD-31) were determined. The expressions of transforming growth factor β 1 (TGF- β 1), p-smad2/3, matrix metalloproteinase-9 (MMP-9) and osteoblast specific transcription factor (osterix) were assayed by western blotting.

Results: The expression of Co1-II in the vertebral body was significantly lower in model mice than in control mice, but was significantly higher in shenggu'an mice when compared with model mice ($p < 0.05$). In shenggu'an mice, Col-I was markedly upregulated, relative to model mice, and the expressions of CD31 in TGF β R IIreceptor inhibitor group and shenggu'an group were lower than in model group ($p < 0.05$). There were significantly lower expressions of TGF- β 1 and p-smad2/3 in the vertebral body of shenggu'an group than in model mice, but osterix was upregulated relative to model mice ($p < 0.05$).

Conclusion: Shenggu'an exerts anti-osteoporotic effect by downregulating TGF β /smad signal pathway. There is thus a potential for its clinical application in the management of osteoporosis.

Keywords: Shenggu'an, TGF β 1-Smad2/3 signal, Bone cartilage metabolism, Osteoporosis

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INTRODUCTION

Vertebral osteoporosis, an osteopathy characterized by low bone mass and destruction of bone tissue microstructure, is the main manifestation in chronic spinal degeneration [1]. There is a steady increase in the number of osteoporosis patients, mostly among postmenopausal women and elderly men, with

bone pain and susceptibility to fracture as the key characteristics [2]. Lumbar injury increases the risk of osteoporosis, and about 25 % of lumbar joint injury occurs in male population, with the incidence positively correlated with age [3].

Vertebral compression fracture caused by osteoporosis is a bone pathology which seriously impairs the well-being of the elderly. Clinical

research shows that traditional Chinese medicine effectively mitigates cervical and lumbar pain caused by intervertebral disc degeneration, and the curative effect is definite [4]. Studies have confirmed that the kidney-tonifying method regulates bone metabolism [5].

Although *shenggu'an* is traditional Chinese medicine which promotes bone formation, there are so far very little investigations on the drug [6]. Transforming growth factor β 1 (TGF- β 1) - Smad2/3 signal participates in the regulation of cartilage metabolism, and maintains stable metabolism and structural integrity of cartilage [7]. Under external stimulation, the cartilage matrix activates TGF- β in the matrix and repairs the tissue. However, it is not in all cases that TGF- β can effectively repair the tissue [8]. Sufficient TGF- β in cartilage formation and development can accelerate cartilage maturation, and low level of TGF- β is needed in mature resting cartilage. Therefore, appropriate amount of TGF- β is the key to maintenance of normal metabolism of cartilage [9].

Advances in osteoporosis research, improvements in medical devices, and the emergence of new drugs have led to a large number of repeatable animal models of osteoporosis. However, there are many limitations in the establishment of this model. There is need to make a model of vertebral osteoporosis in a short period of time. Therefore, this study investigated the anti-osteoporotic effect of *shenggu'an* in relation to the TGF β 1-Smad2/3 signal pathway.

EXPERIMENTAL

Animals and groups

Forty healthy female SPF mice were purchased from Beijing Weitong Lihua Animal Experimental Center (production batch number SCXK (Beijing: 2019-01129). Two groups of mice were used: control mice (sham-operated, 10 mice) and model mice (n = 30). In the control group, the ovaries were exposed without treatment. In the model group, bilateral ovaries and lumbar-to-spine were excised to make the mice lose balance between dynamic and static forces. The model mice were randomly divided into model group, TGF β R II receptor inhibitor group and *shenggu'an* group, with 10 mice in each group. The mice in the control group and model group were given the same volume of normal saline, while those in the TGF β R II receptor inhibitor group were given sb505124 (TGF β R II receptor inhibitor) at a dose of 0.2 ml/10g, twice a day. The *shenggu'an* group received stomach infusion

of concentrated decoction of *shenggu'an* at a dose of 0.2ml/10g, twice a day. After 12 weeks, all mice were sacrificed and L4/5 vertebral bodies were excised.

The study received approval from the Animal Ethical Committee of The Third Affiliated Hospital of Zhejiang Chinese Medical University (approval no. 20197873), and it was carried out in line with the "Principles of Laboratory Animal Care" (NIH article no. 85-23 [10].

Instruments and reagents

The major instruments used, and their sources (in brackets) were: positive fluorescent microscope (Shanghai Wanheng Jingmi Instrument Co. Ltd); automatic tissue dehydrator (Jinhua Shenzhou centrifuge Co. Ltd); paraffin tissue embedding machine (Beijing Keyu Xingye Technology Development Co. Ltd); paraffin tissue slicer (Jinhua Huiyou Instrument Co. Ltd); multifunctional automatic dyeing machine (Beijing dingyong Huatai Technology Co. Ltd); magnetic stirring instrument (Shanghai Yuezhong Instrument Equipment Co. Ltd); constant temperature blast drying oven (Shanghai Binglin Electronic Technology Co. Ltd); toothed tweezers (Shandong shipol Biomedical Co. Ltd); ultrapure water system (Shandong hangaolian Environmental Technology Co. Ltd); enzyme standard instrument (Huatai he Commerce and Trade Co. Ltd); electrophoresis instrument (Beijing Bole Life Science Development Co. Ltd); membrane transfer instrument (Jiangsu Saions Co.); and laser image scanning detection system (Guangzhou Electronic Technology Co. Ltd of Chinese Academy of Sciences).

The reagents used, and their sources (in brackets) were: protein extraction kit (Shanghai Zhennuo Biotechnology Co. Ltd); BCA protein concentration test kit and first antibody diluent (Beijing Solabo Technology Co. Ltd); immunohistochemistry two-part method kit (Shenyang Wanke Biotechnology Co. Ltd); protein electrophoresis molecular weight standard (Beijing taizeruida Technology Co. Ltd); PVDF membrane (Biotechnology Co. Ltd) and ECL luminescent detection kit (Xi'an Dongao Biotechnology Co. Ltd).

Immunohistochemical assay

The L4/5 segment tissues of mice in each group were decalcified, dehydrated, embedded and sectioned to slices of thickness 4 μ m which were soaked in water at 40 °C. The sections were then oven-dried, dewaxed in xylene, dehydrated with

gradient alcohol, and put into EDTA antigen repairing solution at 90 °C. This was followed with washing in distilled water and immersion in 3 % H₂O₂. After rinsing with PBS solution, they were incubated overnight with the primary antibodies Co1-II (1:400), Co1-1 (1:400), and vascular endothelial growth factor (VEGF, 1:400) at -4 °C, followed by incubation with secondary antibody at room temperature for 20 min. The expressions of type II collagen (Co1-1) and platelet endothelial cell adhesion factor (CD31) were examined under the microscope and recorded.

Western blotting

Total protein in the vertebrae of mice was extracted, and after determination of the protein concentration with BCA assay kit, the sample protein was transferred to Heart EP tube and subjected to SDS polyacrylamide gel electrophoresis. The bands were transferred to PVDF membrane which was blocked with skim milk solution for 1 h. The membrane was then incubated with primary antibodies for p-smad2/3, matrix metalloproteinase-9 (MMP-9) and osteoblast specific transcription factors (osterix). This was followed with incubation with secondary antibody at room temperature. The relative protein expressions were determined using chemiluminescence.

Statistical analysis

Results are presented as mean ± SD. Comparison between two groups was performed with *t*-test. Multiple group comparison was done with one-way ANOVA and LSD test. Statistical significance was assumed at $p < 0.05$. The data were analyzed using SPSS 16.0 (IBM, USA).

RESULTS

Expression of Co1-II in vertebrae of mice

The expression of Co1-II in the vertebral body of model mice was markedly lower than that in control mice, but was significantly higher in the vertebral body of the *shengu'an* group than in model mice ($p < 0.05$). The expression level of Co1-II was comparable between TGFβR II receptor inhibitor and model groups ($p > 0.05$). These results are shown in Figure 1.

Co1-1 expression in vertebrae of mice

The expression of Co1-1 in model mice was marked increased, when compared to control mice, while Co1-1 expression in TGFβR II receptor inhibitor mice was markedly reduced,

relative to model mice ($p < 0.05$). Moreover, Co1-1 expression in *shengu'an*-treated mice increased, relative to that in model mice ($p < 0.05$), as shown in Figure 2.

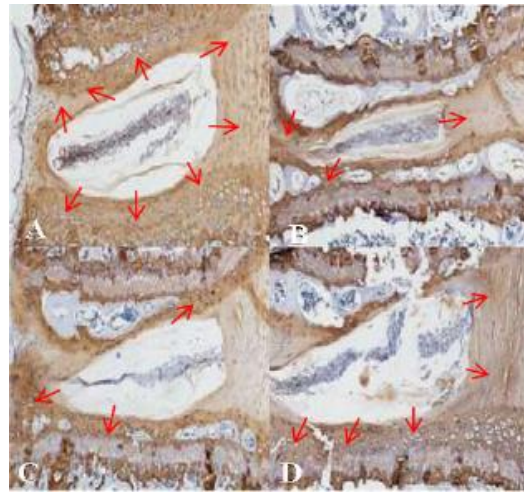


Figure 1: Co1-II expressions in vertebrae of mice among the groups. A: control mice; B: model mice; C: TGFβR II receptor inhibitor mice; D: *shengu'an* mice

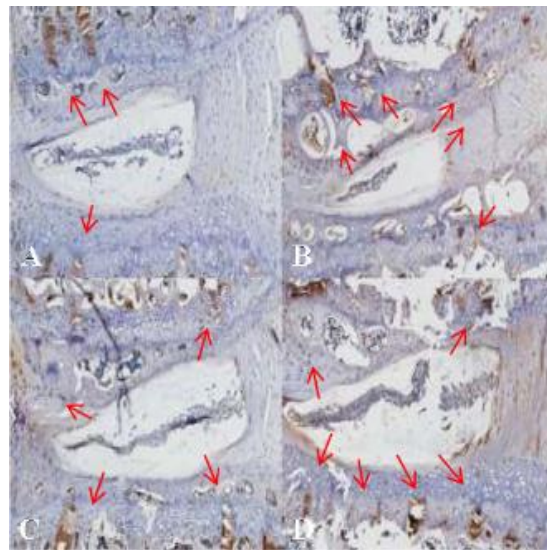


Figure 2: Expression of Co1-1 in vertebrae of mice among the groups. A: Control mice; B: Model mice; C: TGFβR II receptor inhibitor mice; D: *shengu'an* mice

CD-31 expression in vertebrae of mice

As shown in Figure 3, the expression of CD-31 in model group was markedly higher than that in control mice, but was significantly lower in TGFβR II receptor inhibitor and *shengu'an* groups than in model mice ($p < 0.05$).

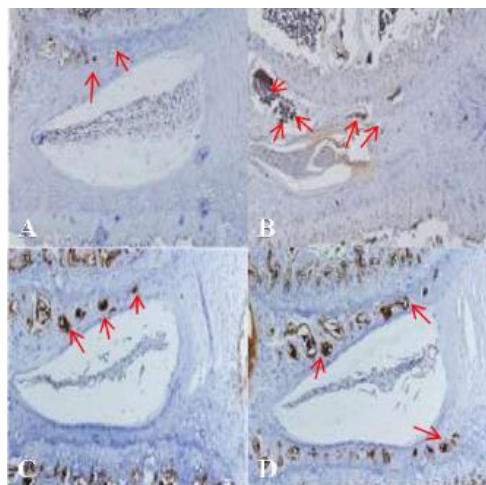


Figure 3: CD31 expression in vertebrae of mice among the groups. A: control mice; B: Model mice; C: TGFβR II receptor inhibitor mice; D: *shenguan* mice

Expressions of related proteins in TGF-β1/smad pathway in vertebrae of mice

The expressions of p-smad2/3, TGF-β1 and MMP-9 in the vertebral body of the model group were significantly higher than those of the control group ($p < 0.05$). Moreover, the expressions of p-smad2/3, TGF-β1 and osterix in the vertebrae of the mice in the TGFβR II receptor inhibitor group were markedly upregulated, relative to model mice ($p < 0.05$). The level of expression of MMP-9 was comparable between mice in TGFβR II receptor inhibitor and model groups. However, p-smad2/3 and TGF-β1 were markedly downregulated in the vertebral body of *shenguan* group, while osterix expression was markedly upregulated, relative to model mice ($p < 0.05$). The expression level of MMP-9 in vertebral body was similar in *shenguan* and model groups ($p > 0.05$). These results are shown in Figure 4.

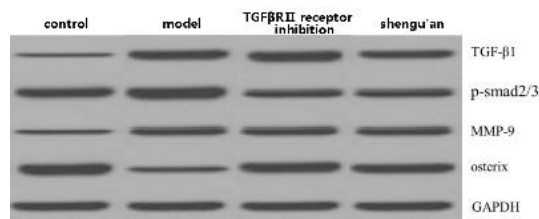


Figure 4: Expressions of related proteins in TGF-β1/smad pathway in vertebrae of mice

DISCUSSION

The vertebral endplate is a semi permeable membrane barrier which connects the intervertebral disc and adjacent upper and lower

vertebrae. It is involved in mechanical conduction. Moreover, it is a key channel for gathering nutrients and excreting wastes. If the structure and function of the vertebral endplate are compromised, degenerative diseases of the intervertebral disc result [11]. The control of mechanical force on the metabolism of osteochondral is realized by mechanical conduction. Changes in mechanical stimulation cause abnormal activation of the molecular pathway of osteochondral cells, leading to related pathological changes [12].

It has been reported that TGFβ1/smad signaling pathway plays an important role in the pathological remodeling of articular cartilage in the OA mice model of joint imbalance. Inhibition of TGFβ1/smad signaling pathway significantly suppresses subchondral bone remodeling caused by joint imbalance, and protects articular cartilage [13]. The content of TGF-β1 in the embedded bone matrix is very high. If pathological bone remodeling occurs, a large number of TGF-β1 will be produced, which will lead to the damage of subchondral bone [14].

Shenguan is a popular Chinese medicine used for clinical prevention and treatment of intervertebral disc degeneration. Some studies have shown that *Yiqi Huayu Bushen* recipe reduces the clinical symptoms in patients with cervical disc degeneration. Other studies have shown that *shenguan*, a Chinese medicine for tonifying the kidney, regulates bone metabolism and bone remodeling. Many studies have confirmed that kidney-tonifying herbs affect the activation of TGFβ1/smad pathway [15]. The TGFβ1/bone morphogenetic protein (BMP) pathway is similar to balance in kidney *yin and yang* in traditional Chinese medicine, indicating that kidney tonifying Chinese medicine for improving bone metabolism may be based on the TGFβ1/smad pathway, and so may be beneficial in the prevention and treatment of vertebral osteoporosis [16].

In model mice, the expression of Co1-II in the vertebral body was markedly downregulated, relative to control, but it was markedly increased in the *shenguan*-treated mice, relative to model mice. This suggests that *shenguan* promoted the expression of Co1-II. The expression of Co1-1 was markedly upregulated in model mice, relative to control, but it was significantly lower in TGFβR II receptor inhibitor mice than in model mice. There was higher level of Co1-I in *shenguan*-treated mice than in model mice. These results suggest that *shenguan* promoted the expression of Co1-1. The expression of CD-31 was higher in model mice than in control

mice, but was significantly lower in TGF β R II receptor inhibitor and *shenggu'an* groups than in model mice. These results show that *shenggu'an* inhibited vascular erosion.

There were higher expressions of p-smad2/3, TGF- β 1 and MMP-9 in the vertebral body of model mice than those in control mice, and osterix level was markedly lower than that in control mice. The expressions of TGF- β 1 and osterix in the vertebrae of mice in the TGF β R II receptor inhibitor group were markedly increased, while p-smad2/3 expression was downregulated, relative to model mice. Moreover, p-smad2/3 and TGF- β 1 were markedly downregulated in vertebral body of *shenggu'an*-treated mice, while osterix expression was markedly upregulated, relative to model mice. These results indicate that inhibition of osteoclast significantly suppressed the production of TGF- β 1, leading to downregulation of the TGF- β 1-Smad2/3 pathway. *Shenggu'an* regulated the expressions of p-smad2/3, TGF- β 1 and MMP-9 in TGF β /smad signal pathway, and inhibited the TGF β /smad signal pathway. The inhibition of the signal pathway and osteoclast mitigated osteoporosis. The effect of *shenggu'an* was similar to that of TGF β 1 receptor inhibitor, to a certain degree.

These results indicate that the TGF β /smad signal pathway which was activated in osteoporosis, was inhibited by *shenggu'an*. Thus, *shenggu'an* may be effective in the treatment of osteoporosis.

CONCLUSION

Shenggu'an exerts an anti-osteoporotic effect through downregulation of the TGF β /smad signal pathway. Therefore, it has potential for clinical application in the treatment of osteoporosis.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Authors' contributions

This study was done by the authors named in this article, and the authors accept all liabilities resulting from claims which relate to this article and its contents. The study was conceived and designed by Xinmiao Yao; Wei Li, Zhiqiang Peng, Yulun Wu, Jintao Hu, Peilun Li, Xinmiao Yao collected and analyzed the data; Wei Li wrote the manuscript. All authors read and approved the manuscript for publication.

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REFERENCES

1. Watts NB, Diab DL. Long-term use of bisphosphonates in osteoporosis. *J Clin Endocrinol Metab* 2010; 95: 1555-1565.
2. Chen X, Su JC. New focus on osteoporosis: Differentiation fate of bone marrow-derived mesenchymal stem cells. *Acad J Second Mil Med University* 2017; 38: 397-404.
3. Xu X, Jia X, Mo L, Liu C, Zheng L, Yuan Q, Zhou X. Intestinal microbiota: a potential target for the treatment of postmenopausal osteoporosis. *Bone Res* 2017; 5: 17046-17058.
4. Gui SQ, Yu J, Wei MJ. Experimental study on effect of tonifying kidney herbs on pituitary, ovary, and adrenal gland in androgen sterilized rats. *Chin J Integr Tradit Western Med* 2017; 3: 189-193.
5. Li W, Zhang X, Wang J, Li M, Cao C, Tan J, Ma D, Gao Q. TGF β 1 in fibroblasts-derived exosomes promotes epithelial-mesenchymal transition of ovarian cancer cells. *Oncotarget* 2017; 8: 96035-96047.
6. Wei MJ, Yu J. Effect of kidney tonifying herbs on morphological changes of adrenal cortex in androgen-sterilized rats. *Zhongguo Zhongxiyi Jiehe Zazhi* 2018; 12: 736-738.
7. Zhang H, Hu J, Liu L. MiR-200a modulates TGF- β 1-induced endothelial-to-mesenchymal shift via suppression of GRB2 in HAECs. *Biomed Pharmacother* 2017; 95: 215-222.
8. Li Y, Zhu H, Wei X, Li H, Yu Z, Zhang H, Liu W. LPS induces HUVEC angiogenesis in vitro through miR-146a-mediated TGF- β 1 inhibition. *Am J Transl Res* 2017; 9: 591-600.

9. Yu Y, Duan J, Li Y, Li Y, Jing L, Yang M, Wang J, Sun Z. Silica nanoparticles induce liver fibrosis via TGF- β 1/Smad3 pathway in ICR mice. *Int J Nanomedicine* 2017; 21: 6045-6057.
10. World Health Organization. Principles of laboratory animal care. *WHO Chron* 1985; 39: 51-56.
11. Zhu LG, Zhang P, Song QH, Zhang WQ, Zhu HW, Yang LP, Zhu Y, Wang Y, Li LH. Preliminary study of intervention in effect of Bushen Huoxue recipe on calcification of lumbar vertebra cartilage endplate of the aging gerbils. *Zhongguo Gu Shang* 2017; 30: 926-932.
12. More N, Kapusetti G. Piezoelectric Material - A Promising Approach for Bone and Cartilage Regeneration. *Med Hypotheses* 2017; 108: 10-16.
13. Yeh HW, Hsu EC, Lee SS, Lang YD, Lin YC, Chang CY, Lee SY, Gu DL, Shih JH, Ho CM, et al. PSPC1 mediates TGF- β 1 autocrine signalling and Smad2/3 target switching to promote EMT, stemness and metastasis. *Nat Cell Biol* 2018; 20: 479-491.
14. Li Y, Liu H, Liang Y, Peng P, Ma X, Zhang X. DKK3 regulates cell proliferation, apoptosis and collagen synthesis in keloid fibroblasts via TGF- β 1/Smad signaling pathway. *Biomed Pharmacother* 2017; 91: 174-180.
15. Ma F, Li W, Liu C, Li W, Yu H, Lei B, Ren Y, Li Z, Pang D, Qian C. MiR-23a promotes TGF- β 1-induced EMT and tumor metastasis in breast cancer cells by directly targeting CDH1 and activating Wnt/ β -catenin signaling. *Oncotarget* 2017; 8: 69538-69550.
16. Tan WJ, Tan QY, Wang T, Lian M, Zhang L, Cheng ZS. Calpain 1 regulates TGF- β 1-induced epithelial-mesenchymal transition in human lung epithelial cells via PI3K/Akt signaling pathway. *Am J Transl Res* 2017; 9: 1402-1409.