Effects of *Bushen Huogu* decoction on bone metabolism of ovariectomized osteoporotic rats★

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Abstract

BACKGROUND: *Bushen Huogu* decoction can effectively prevent and treat osteoporosis, but the concrete mechanism of pharmacology is still not clear. 25-hydroxy vitamin D3 and 1, 25-dihydroxyvitamin D3 are important coupling factors, which can regulate bone resorption and formation.

OBJECTIVE: To investigate the curative effects of *Bushen Huogu* decoction on the bone mineral density, bone biomechanics, and level of 25-hydroxy vitamin D3 and 1, 25-dihydroxyvitamin D3 in blood serum, liver and kidney in the ovariectomized osteoporotic rats.

METHODS: Totally 108 healthy female Sprague-Dawley rats were randomly divided into model group, sham-operated group, and treatment group. All rats had been ovariectomized to induce estrogen absence and further establish osteoporotic models, except those in sham-operated group. Treatment group of rats were intragastrically administrated with 2 mL *Bushen Huogu* decoction, twice a day.

RESULTS AND CONCLUSION: Compared with model group, the bone mineral density in the rat femur was significantly increased in the treatment group ($P < 0.05$), the index of maximal stress and maximal loading of the femoral head were also increased ($P < 0.05$). The concentration of 25-hydroxy vitamin D3 and 1, 25-dihydroxyvitamin D3 in blood serum, liver and kidney were significant higher in the treatment group than those in the model group, and the levels were similar with those in sham-operated group ($P > 0.05$). In the early period of estrogenic hormone absence, the Chinese kidney-tonifying drugs could activate bone metabolism, raise bone mineral density and reinforce quality of bone through up-regulating expression of 25-hydroxy vitamin D3 and 1, 25-dihydroxyvitamin D3.

INTRODUCTION

Nowadays, osteoporotic fractures are one of the most important public health problems encountered over 30 years of age, especially in older women, with high morbidity, mortality and treatment costs[1]. The involvement of vitamin D and its endocrine system are essential both for the process of bone development and growth, as well as bone remodeling. Important bone cells participating in those processes include the osteoblast (bone formation), the osteoclast (bone resorption), and the growth plate chondrocyte (longitudinal bone growth). Serum 1, 25-dihydroxyvitamin D3 [1, 25-$(OH)_2D_3$] plays a vital role throughout the session and it is sequentially synthesized in the skin, liver and kidney. This experiment was aimed to investigate the effects of *Bushen Huogu* decoction on the level of 5-hydroxy vitamin D3 [25-$(OH)D_3$] and 1, 25-$(OH)_2D_3$ in blood serum, liver and kidney, as well as bone mineral density (BMD) and biomechanics in the ovariectomized osteoporotic rats, in a broader attempt to explore the mechanism of treating osteoporosis with traditional Chinese medicines.

MATERIALS AND METHODS

Design

A completely randomized controlled study was conducted.

Time and setting

This experiment was carried out in the Bone Metabolism Laboratory of Chinese and Western Medicine (Level 3 Laboratory of the National Administration of Traditional Chinese Medicine), Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, China from May 2010 to January 2011.

Materials

Animals

Totally 108 female Sprague-Dawley rats, with a mean weight of (380±10) g, aged 8 months, were provided by the Center of Animal Experiment, Tongji Medical College, Huazhong University of Science and Technology, China.

Drugs

Bushen Huogu decoction (mainly composed of Chinese herbs such as *Herba Epimedii* and *Cortex Eucommiae*; 10 mL/bottle; State Medical Permitment No. Z20000039; Production Batch No. 20030002) were donated by Xinjiang Huashidan Pharmaceutical Co., Ltd., China.

The main reagents and apparatus are introduced as follows:

Reagent and instrument	Source
25-hydroxy vitamin D kit (item code: NZ-003, IDS) and 1, 25-hydroxyvitamin D kit (item code: NZ-004, IDS)	IDS Co., Ltd, UK
Dual energy X-ray absorptiometry (type Hologic 2000 Plus)	Hologic, Mass, USA
Biomaterial testing machine (AGS-H10KN)	AUTOGRAPH, Japan

Methods

Grouping management

108 Sprague-Dawley rats were randomly divided into

three groups: model, sham-operated, and treatment. Each group contained 36 rats.

Modeling

Rats were anesthetized *via* an intraperitoneal injection of 2.5% thiopental sodium (2 mL/kg body weight). The rat models of osteoporosis in model group and treatment group were established by excising two ovaries from dorsal incision, while the same size of fat was excised in the sham-operated group. All rats were given an intramuscular injection of penicillin (80 000 U/d) for 3 days, to prevent infection.

Intervention

According to human body frequently used dosage 20 g/kg and medicine conversional method between human being and animals, rats in the treatment group were treated with 2 mL *Bushen Huogu* decoction *via* intragastric administration, twice a day. Normal saline was administrated intragastrically into the rats of other two groups at the coordinative capacity and same frequencies.

Specimen collection

A total of 36 rats (12 in each group) were killed respectively at 4, 8, 12 weeks after operation, and the intact right femora head were got carefully by removing away all tissues adherent. The femoral head were prepared for BMD and biomechanical testing, stored at −70 ℃. The rats' serum was isolated and collected routinely. A small part of tissue samples of liver and kidney were collected, with saline to wash miscellaneous, snipped, weighed, and added together with 5 × phosphate buffered saline into the homogenizer. Rotated handle for appropriate time. The tube was submersed in an ice bath to maintain the sample at 4 ℃. Then the homogenate liquid was removed into the 5-mL centrifuge tube centrifuged at 4 000 r/min for 20 minutes at 4 ℃. Then supernatant was removed into another tube centrifuged at 10 000 r/min for 10 minutes at 4 ℃ and the supernatant was stored at −70 ℃ prepared for determination of the quantitative of 25-$(OH)D_3$ and 1, 25-$(OH)_2D_3$.

Measurements of BMD

The right hip femoral neck of rats were measured by dual energy X-ray absorptiometry using a Hologic QDR 2000 plus. Procedures of determination selected small animal software scans and subregion analysis of right hip scans. All BMD results were expressed as g/cm^2. Results automatically printed from printer equipment which attached to the apparatus. The precision of the dual energy X-ray absorptiometry was evaluated over three repeated measurement[2] of different BMD values in subjects, giving a mean coefficient of variation (range: 0.33%–0.40%). After the measurement, femoral head stored at −80 ℃ with saline gauze parceled until biomechanical properties characteristics detected.

Measurements of biomechanical index

The freezing specimens were put on the table at room temperature until ice was melt completely. Then they were loaded horizontally on the Biomaterial Testing Machine and undergone three-point bending test. The loading points were located in the middle parts of specimens; the span between two points was 2.2 cm and loading rate 5 mm/min. The loading-deformation curve and the maximal breaking force were printed. Load-displacement curve was normalized by cross-sectional area and this curve was converted to a stress-strain curve. Stress was determined from the stress-strain curve and it represents the maximum stress before fracture occurred. The maximal stress was calculated from the equation:

s=F/A.

s is the maximal stress (MPa), F is the failure load (N), and A is the cortical area of the specimen (m^2).

Concentrations of 1, 25-$(OH)_2D_3$ and 25-$(OH)D_3$ as detected by enzyme-linked immunosorbent assay

Samples were centrifuged at 2 000 *g* for 15 minutes and 100 μL of delipidated sample was utilized for Immunocapsules SORB in duplicate. Replace caps securely. Immunocapsules SORB was placed in foam rack and rotated end-over-end at 5–20 revolutions per minute for 90 minutes at room temperature (18–25 ℃). Immunocapsules SORB was standing upright in foam rack for 3–5 minutes to allow gel to settle. Any gel adhering to the screw caps was dislodged. Gel was allowed to settle for additional 1–2 minutes. Screw cap was removed and break off (do not twist off) bottom stopper from Immunocapsules SORB and Immunocapsule SORB was placed in a plastic tube. Samples were centrifuged at low speed (500–1 000 *g*) for approximately 1 minute to remove sample. 500 μL of deionised water was added to each Immunocapsule SORB and the operation should be careful to avoid water splashing out of the Immunocapsule SORB. Samples were centrifuged at a low speed (500–1 000 *g*) for approximately 1 minute to wash immunoextraction gel. The above wash step was repeated twice. The labeled borosilicate glass tubes were prepared for each Immunocapsule SORB, which was transferred to the glass tubes. 150 μL of Elution Reagent REAG 2 was added to all Immunocapsules SORB. All reagents were soaked in solid phase for 1–2 minutes. Samples were centrifuged at low speed (500–1 000 *g*) for approximately 1 minute to collect eluate. The above steps were repeated twice. The total elution volume collected is therefore 450 μL for each sample. Immunocapsules SORB was discarded and tubes were placed in a heating block or water bath at 40 ℃. The eluates were evaporated under a gentle flow of nitrogen. Evaporation should last 20–30 minutes. And we should ensure no remaining liquid in the tubes. 100 μL of Assay Buffer was added to each tube and residues were dissolved. The immunopurified samples are now ready for assay.

100 μL of each Calibrator CAL was added to the appropriately labeled tubes and pipette directly to the bottom of the tube. Sample was collected from the tubes extracted from the above steps. 100 μL of primary antibody solution was added to all tubes. All tubes were shaken gently without foaming. Samples were incubated at 2–8 ℃ overnight (16–20 hours). 150 μL of the solution was added to the culture wells of the antibody-coated plate (MICROPLAT). The first two wells that were empty served as the blanks. The plate was covered with an adhesive plate sealer and incubated on an orbital shaker (500–750 r/min) at 18–25 ℃ for 90 minutes. 100 μL of 1, 25D Biotin solution was added to all wells except the blanks. The

plate was covered with an adhesive plate sealer and incubated on an orbital shaker (500–750 r/min) at 18–25 ℃ for 60 minutes. All wells were washed three times with wash solution. 200 μL of Enzyme Conjugate (ENZYMCONJ) was added to all wells except the blanks using a multichannel pipette. The plate was covered with an adhesive plate sealer and samples were incubated at 18–25 ℃ for 30 minutes. Wash step was repeated as above. 200 μL of TMB Substrate was added to all wells including the substrate blanks using a multichannel pipette. The plate was covered with an adhesive plate sealer and samples were incubated at 18–25 ℃ for 30 minutes. 100 μL of Stop Solution HCl was added to all wells using a multichannel pipette. The absorbance of each well at 450 nm was read using a microplate reader within 30 minutes after adding the Stop Solution.

The percent binding (B/Bo%) of each calibrator, control and unknown sample will be calculated as follows:

$$B/Bo\% = \frac{(\text{mean absorbance} - \text{mean absorbance substrate blank})}{(\text{mean absorbance for '0' cal.} - \text{mean absorbance substrate blank})} \times 100\%$$

A calibration curve was prepared on semi-log graph paper by plotting B/Bo% on the ordinate against concentration of 1, 25-$(OH)_2D_3$ or 25-$(OH)D_3$ on the abscissa. The B/Bo% for each unknown sample was calculated and read values off the curve in pmol/L or in nmol/L. Calculates results using MultiCalc (PerkinElmer) data reduction software with a 4PL curve fit plotting net absorbance versus log concentration [3].

Main outcome measures

The levels of 25-$(OH)D_3$ and 1, 25-$(OH)_2D_3$ in blood serum, liver and kidney in ovariectomized osteoporosis rats, the BMD of the femoral head and biomechanical properties characteristics of the bone.

Statistical analysis

Statistical analysis was made with SPSS 13.0 software. The results were expressed as mean±SD. Comparisons were made by one-way analysis of variance. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Quantitative analysis of experimental animals

All 108 rats were involved in the final analysis without any drop-out.

Levels of 25-$(OH)D_3$ and 1, 25-$(OH)_2D_3$

At 4–12 weeks after surgery, the concentration of 25-$(OH)D_3$ in blood serum and liver and the concentration of 1, 25-$(OH)_2D_3$ in blood serum and kidney were expressed increasingly in both sham-operated group and treatment group, with no significant difference ($P > 0.05$). But the levels were significantly higher than that in model group ($P < 0.05$). The concentration of 25-$(OH)D_3$ was significantly increased at 8 and 12 weeks, compared with 4 weeks ($P < 0.05$). While the concentration of 1, 25-$(OH)_2D_3$ was increased slowly, and there were significant differences between 4 weeks and 12 weeks ($P < 0.05$; Table 1).

Table 1 The concentration of 25-hydroxy vitamin D3 [25-$(OH)D_3$] in blood serum and liver and the concentration of 1, 25-dihydroxyvitamin D3 1, [25-$(OH)_2D_3$] in blood serum and kidney ($\bar{x}$±*s*, *n*=12)

Item	Model group	Treatment group	Sham-operated group
Concentration of 25-$(OH)D_3$ in serum(μg/L)			
4 wk	2.89±0.02	7.91±4.06[a]	13.22±6.49[a]
8 wk	4.83±1.92[b]	10.19±4.22[ab]	13.30±6.21[ab]
12 wk	5.44±2.28[b]	16.20±1.29[ab]	14.41±2.86[ab]
Concentration of 25-$(OH)D_3$ in liver tissue (μg/L)			
4 wk	0.41±0.39	0.79±0.46[a]	0.90±0.77[a]
8 wk	0.39±0.22	1.09±0.73[ab]	1.18±0.67[ab]
12 wk	0.34±0.12	1.17±0.61[ab]	0.96±0.35[a]
Concentration of 1, 25-$(OH)_2D_3$ in serum (ng/L)			
4 wk	82.20±38.19	124.40±53.86[a]	115.80±4.60[a]
8 wk	76.49±30.61	119.72±8.98[a]	125.39±20.50[a]
12 wk	61.94±8.61	145.01±37.0[a]	141.93±60.96[a]
Concentration of 1, 25-$(OH)_2D_3$ in kidney tissue (ng/L)			
4 wk	95.18±41.85	124.55±4.16[a]	123.88±13.14[a]
8 wk	89.27±18.73	129.61±48.64[a]	128.54±17.34[a]
12 wk	89.12±17.99	145.27±57.62[a]	143.49±38.60[a]

[a]$P < 0.05$, *vs.* model group; [b]$P < 0.05$, *vs.* 4 wk

Results of biomechanical properties

Through the three-point bending test, the curative effects of *Bushen Huogu* decoction on the biomechanical properties of femoral head in ovariectomized rats were detected. Results showed that biomechanical properties of the femoral head were deteriorated in model group. They were changed obviously in the index of maximal stress and maximal loading, which were lower than the sham-operated group ($P < 0.05$). On the contrary, the index of biomechanical properties of the femoral head was restored in both sham-operated and treatment group, and there was no significant difference between the two groups ($P > 0.05$).

Table 2 Results of biomechanical properties of the femoral head in rats ($\bar{x}$±*s*, *n*=12)

Item	Model group	Treatment group	Sham-operated group
Maximal stress (N)			
4 wk	280.26±23.33	315.28±24.36[a]	333.25±39.63[a]
8 wk	272.26±24.55	318.57±26.68[a]	332.95±35.55[a]
12 wk	270.17±25.36	321.42±53.33[a]	337.26±41.48[a]
Maximal loading (N/mm^2)			
4 wk	32.33±4.36	37.85±4.65[a]	38.65±5.32[a]
8 wk	31.24±5.12	37.89±5.23[a]	39.98±5.31[a]
12 wk	30.22±4.97	38.15±5.45[a]	41.52±6.36[a]

[a]$P < 0.05$, *vs.* model group

Results of BMD

At 4–12 weeks after modeling, the BMD of the femoral head in model group was lower than that in sham-operated group ($P < 0.05$). The BMD of treatment group and sham-operated

group were both increased and there were no significant difference between the two groups ($P > 0.05$).

Table 3 Results of bone mineral density of femoral head in rats ($\bar{x}$±s, n=12, g/cm^2)

Time	Model group	Treatment group	Sham-operated group
4 wk	0.281±0.012	0.301±0.017[a]	0.308±0.014[a]
8 wk	0.279±0.015	0.305±0.014[a]	0.310±0.018[a]
12 wk	0.276±0.016	0.306±0.019[a]	0.312±0.016[a]

[a]$P < 0.05$, *vs.* model group

DISCUSSION

Osteoporosis, one of the major and growing health problems around the world, is defined as a systemic skeletal disease characterized by low bone mass and micro-architectural deterioration of bone tissue, with high bone fragility and susceptibility to fractures. In this experiment, ovariectomized rats showed many characteristics similar with postmenopausal osteoporosis, ad it is recognized one of the most reliable small animal model of postmenopausal osteoporosis[4]. Bone biomechanical which is used to study the mechanical properties and the biological effects of bone, basing on engineering mechanics, is one of the most reliable indicators of evaluating comprehensive of bone quality[5-14]. BMD is recognized as the most significant measurable determinant of bone strength as well as one of the most important risk predictors for osteoporosis fracture (one of the most serious complication of osteoporosis)[15-20].

The vitamin D endocrine system is critical for the proper development and maintenance of mineral ion homeostasis and skeletal integrity, most studies still focus on it[21-25]. The hormonal or bioactive form of vitamin D is 1,25-$(OH)_2D_3$. It is generated from sequential hydroxylations of vitamin D3, a secosteroid precursor that is obtained from the diet or produced in the skin upon exposure to UV light. The first hydroxylation of vitamin D3 occurs at the C-25 position and is catalyzed by vitamin D-25-hydroxylase in the liver to produce 25-$(OH)D_3$, the major circulating form of vitamin D in mammals. 25-$(OH)D_3$ is the substrate for a second hydroxylase, the renal 25-$(OH)D_3$-1α-hydroxylase, resulting in the production of the most bioactive metabolite 1,25-$(OH)_2D_3$. 1,25-$(OH)_2D_3$ is one of the major regulators of calcium (and phosphate) metabolism, stimulating intestinal calcium absorption. However, it also may have direct effects on the bone[26], in which continuous remodeling must occur to sustain structural integrity.

It is confirmed that there was a close relationship between serum 1,25-$(OH)_2D_3$ levels and BMD in animals[27]. Previous studies suggested that vitamin D_3 (> 700 IU/d) with calcium supplementation compared to placebo has a small beneficial effect on BMD, and reduces the risk of fractures. Vitamin D receptor mediates the role of 1,25-$(OH)_2D_3$ in the process of absorption of calcium and phosphorus in the intestinal and bone mineralization role. The vitamin D receptor-knockout mice are viable and develop normally until the weaning period[28]. However, shortly after weaning, vitamin D receptor-null mice exhibit alopecia and growth retardation accompanied by progressive hypocalcemia, hypophosphatemia, and compensatory hyperparathyroidism. These metabolic imbalances result in severe skeletal defects, including decreased bone mineral density, thinned bone cortex, and widened undermineralized growth plates. So by detecting serum 1,25-$(OH)_2D_3$ can evaluate the level of calcium absorption levels in order to diagnosis and monitor of osteoporosis in early period.

In this experiment, we utilized *Bushen Huogu* decoction as experimental drugs and the subscription was mainly composed of Chinese herbs such as donkey-hide glue, prepared rhizome of rehmannia, barbary wolfberry fruit, and so on. Then concentrations of 25-$(OH)D_3$ and 1,25-$(OH)_2D_3$, the index of biomechanical properties, and the BMD was increased quickly in 4 weeks and increased slowly in 8 and 12 weeks. It indicated that *Bushen Huogu* decoction could effectively promoted concentration of 25-$(OH)D_3$ and 1, 25-$(OH)_2D_3$ and increased BMD early.

Previous studies suggest that *Bushen Huogu* decoction was effective in preventing and treating osteoporosis[29]. The partial mechanism may be associated with the fact that *Bushen Huogu* decoction can promote cellular proliferation of osteoblasts and regulating osteoprotegerin/the receptor activator of nuclear factor-κB ligand expression[30]. Recent studies confirmed that *Bushen Huogu* decoction could prevent and treat bone loss through the roles of some cytokines, such as vascular endothelial growth factor and fibroblast growth factor-2 in the bone tissues and cells, which could accelerate intracartilaginous ossification and intramembranous ossification to promoting preosteoblast and chondroblast caryocinesia, blood vessel growth and osseous anabolism[31].

This study showed that in the early period in absence of estrogenic hormone, *Bushen Huogu* decoction could activate bone metabolism, raise BMD, reinforce quality of bone and up-regulate the expressions of 25-$(OH)D_3$ and 1, 25-$(OH)_2D_3$ at molecular levels. The findings indicate that *Bushen Huogu* decoction have a definite antiosteoporotic effect.

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中药补肾活骨方对去势骨质疏松大鼠骨代谢的影响*

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摘要

背景：中药补肾活骨方可有效防治骨质疏松症，但其具体的药理学机制仍不是很清楚。25-羟基维生素 D3 和 1，25-二羟基维生素 D3 是调节骨吸收与骨形成的重要的偶联因子。

目的：观察补肾中药对去势骨质疏松大鼠骨密度、骨生物力学、血清及肝肾组织中 25-羟基维生素 D3 和 1，25-二羟基维生素 D3 水平的影响。

方法：健康雌性 SD 大鼠 108 只随机等分为假手术组、模型组和治疗组。后 2 组摘除双侧卵巢，导致雌激素缺失，从而诱导骨质疏松症模型。治疗组大鼠造模后以中药补肾活骨方 2 mL 灌胃，2 次/d。

结果与结论：与模型组相比，治疗组股骨头骨密度明显提高($P < 0.05$)，最大应力和最大负荷指数明显增强($P < 0.05$)，血液、肝脏和肾脏组织中 25-羟基维生素 D3 和 1，25 二羟基维生素 D3 水平明显提高($P < 0.05$)；且接近于假手术组($P < 0.05$)。提示补肾中药在雌激素缺失早期即可在分子水平上调节 25-羟基维生素 D3 和 1，25-二羟基维生素 D3 的表达水平，激活骨代谢提高骨密度增强骨质量达到预防骨质疏松的作用。

关键词：补肾活骨方；1，25-二羟基维生素 D3；骨密度；骨生物力学；骨质疏松症；骨代谢

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