Basic Investigations

Effects of Zuogui Pill (左归丸) on Wnt Singal Transduction in Rats with Glucocorticoid-induced Osteoporosis

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Objective: To reveal the mechanism of Zuogui Pill (左归丸) in treatment of glucocorticoid-induced osteoporosis from the angle of the Wnt signal transduction pathway and to provide further experimental evidence for expounding the scientific connotation of "the kidney dominating the bones" in TCM.
Methods: Forty-two male Wistar rats were selected and randomly divided into three groups, control group (*n*=12), model group (*n*=15) and Zuogui Pill group (*n*=15). Form the beginning, The rats were injected dexamethasone for eight weeks to make the model of osteoporosis, and the Zuogui Pill were administered intragastrically to the rats of Zuogui Pill group for eight weeks. The relative morphological parameters were measured in the undecalcified tibial slices. And the protein expression levels of Wnt1, LRP-5 and ß-catenin in rat tibial osteoblasts (OB) and bone marrow stromal cells (BMC) were detected by immunohistochemistry.
Results: Compared with the control group, TBV% and TFS% decreased significantly, while TRS% increased significantly, and the protein expression of Wnt1, LRP-5 and ß-catenin in OB and BMC decreased significantly in the model group. And compared with the model group, TBV% and TFS% increased significantly, and expression levels of Wnt1, LRP-5 and ß-catenin proteins increased significantly in the Zuogui pill group.
Conclusion: Zuogui Pill can prevent and treat glucocorticoid-induced osteoporosis in rats by up-regulating the expression of the key signal molecules Wnt1, LRP-5 and ß-catenin in Wnt signal transduction pathway.

Keywords: *Zuogui Pill; osteoporosis; osteoblast; Wnt; LRP; ß-catenin*

Normal bone metabolism depends on the balance state between bone formation induced by osteoblast and bone absorption caused by osteoclast. If the bone absorption is over the bone formation, the bone amount will reduce, severe reduction of the bone amount would lead to osteoporosis generation. Osteoblasts are important cells directly participating in bone metabolism in bone marrow micro-environment, which play extremely important role in whole course of development, formation and reconstruction of the bone tissues, being very important for regulation of differentiation and development of osteoblasts. In resent years, studies indicate that Wnt signal transduction is a key pathway for cell development and regulation of ell growth, and plays a key role in orientation, differentiation, development and proliferation of osteoblasts, being a very important regulative pathway of bone metabolism;[1-3] Wnt, LRP-5 and ß-catenin are a key link in this pathway. In the study, the glucocorticoid-induced osteoporosis rat model was replicated and effects of Zuogui Pill (左归丸) on histomorphological measurement parameters and the expression levels of Wnt1, LRP-5 and ß-catenin proteins in tibial osteoblasts (OB) and bone marrow stromal cells (BMC) were investigated in the rat with glucocorticoid-induced osteoporosis, so as to probe into the mechanism of Zuo Gui Pill in treatment of glucocorticoid- induced osteoporosis and provide further experimental evidence for expounding the scientific connotation of "the kidney dominating the bones".

MATERIALS AND METHODS

Experimental Animal

Forty-two female Wistar rats of sanitary degree, weighing 200–220 g, were supplied by the Center for Experimental Animals, Chinese PLA Academy of Military Medicines, License No：SCXK（Jun）2002—001. The rats were raised in the room for experimental animals of sanitary degree, Institute of Basic Theories, China Academy of Chinese Medical Sciences, License No: SYK11-00-0039.

Drugs

Zuo Gui Pill was composed of Shu Di (Radix Rehmanniae Preparata) 24 g, Shan Yao (Rhizoma Dioscoreae) 12 g, Guo Qi Zi (Fructus Lycii) 12 g, Shan Yu Rou (Fructus Corni) 12 g, Niu Xi (Radix Achyranthis Bidentatae) 9 g, Tu Si Zi (Semen Cuscutae) 12 g, Gui Ban Jiao (Colla Plasti Testutinis) 12 g, Lu Jiao Jiao (Colla Cornus Cervi) 12 g., which were routinely decocted with water into 9.52 g crude drugs/kg with an administration concentration of 0.952 g/mL. Dexamethasone sodium phosphate injection was made in

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Tinjin Jinyao Amino Acid Co. Ltd (batch No: 0704031), specification: 5 mg/mL, which was diluted into 1 mg/mL with saline at use. Administration volume: 0.1 mL/100 g body weight, i.e., 0.1 mg/100 g body weight.

Main Reagents

Rat β-catenin antibody: produced by Cell Signaling Co. , USA; Rat Wnt1 and LRP-5 antibodies: Santa Cruz Company, USA; Antibody II two-step method immuno-histochemical reagent DAB Kit was purchased from Beijing Zhongsan Jinqiao Biotechnique Co. Ltd.; Pentobarbital: Beijing Tong County Yucai Fine Chemical Plant, batch No: 950427; Tetracycline hydrochloride: supplied by Xinhui Zeao Science and Technology Co. Ltd, batch No: US22105; Ethylene diamine tetraacetic acid disodium (EDTA.Na_2): produced by Beijing Beihua Fine Chemicals Co .Ltd, batch No: 20060324; Methyl acrylic succinate: Beijing Yili Fine Chemicals Co. Ltd, batch No: 980716; Polyethylene glycol 400: produced by Beijing Chemical Reagents Co., batch No: 050425; Methyl benzoate: Huabei District Specific Chemical Reagents Development Center (Tianjin)., batch No: 980923; Benzoyl peroxide: Beijing Jinlong Chemical Reagents Co. Ltd, batch No: 20000420；N,N-Dimethyl-p-toluidine: produced by ALDRICH Co. Lot.: 04327MB-235; Solution I: 100 mL methyl acrylic succinate + 35 mL butyl acrylic succinate + 1.2 mL polyethylene glycol 400; Solution II: Solution I + 0.4 g dried benzoyl peroxide; Solution III: Solution II + 0.8 g dried benzoyl peroxide.

Main Instruments

Freezing microtome: TBS Co. UK; Reicheit-Jung 2040 microtome: Germany; LEICA CTR6000 microscope: Leica Co. Germany; Leica-Qwin Image Analyzer System: Leica Co. Germany; Thermostat: Medical Instruments Factory of Shanghai Boshen Industry Co. Ltd.

Grouping and Treatment of Animals

Forty-two female Wistar rats were randomly divided into 3 groups, blank control group (*n*=12), model group (*n*=15) and Zuo Gui Pill group (*n*=15). The rat of the model group was administrated with intramuscular injection of dexamethasone at 0.1 mL/100 g body weight with a concentration of 1 mg/mL, i.e., 1 mL/kg body weight, twice each week, for 8 weeks; The blank control group was injected with the same volume of saline. For the Zuo Gui Pill group, in addition to intramuscular injection of dexamethasone, Zuo Gui Pill decoction was administrated intragastrically at a dose of 9.52 g crude drugs/kg body weight (1 mL/100 g body weight, 0.952 g crude drugs/mL), once each day, for 8 weeks.

The rats were intraperitoneally injected with tetracycline hydrochloride (30 mg/kg body weight) 16 days and 3 days before the rat was killed, respectively, for fluorescence labeling of the bone. After administration, the rats were rapidly suffocated to death after anesthesia, and the right tibia was taken and prepared undecalcified tibial sections for examination of bone tissue histochemical parameters; and the left tibia was taken to make freezing sections to detect expression levels of Wnt1, LRP-5 and ß-catenin proteins of OB and MSC.

Detection of Bone Tissue Histochemical Measure Indexes

Preparation and staining of undecalcified tibia sections: The 1/3 proximal end of the right tibia was taken and fixed with 4% paraformaldehyde, and then the preparation and staining of the undelcalcified section were carried out according to Ju's method[1]: Dehydrated grade by grade with 80%, 95%, 100% alcohol, 2 days each, followed by hyalinization with xylene, 2 days. The sections were immerged respectively into solution I, II and II for 3 days each. Then, 400 μLN, N-Dimethyl-p-toluidine was added into100 mL precooled solution III (4 ℃), which was taken 7 mL and poured into a small penicillin bottle and the bone samples were placed in a same direction into the bottle bottom and the cover was tired and the air in the bottle was extracted with an injector. Then they were placed in a refrigerator at -20 ℃ for about 1 week, becoming into a colorless and transparent hard embedding mass. After the mass was repaired, for each mass, 2 sections of 5 μm-longitudinal undecalcified tibial sections were made, one was stained with toluidine blue and another section for fluorescent observation.

Morphological measure method of bone tissues: Leica-Qwin image analyzer system was used for measure of undecalcified tibail sections. Bone trabecula volume percent (TBV%): the percent of bone trabecula volume accounting for the total volume of the detected bone marrow cavity bone, which is a main index judging bone quantity; Bone trabecula absorption surface percent (TRS%): the percent of the irregular, uneven bone trabecula surface accounting for bone trabecula surface, which can judge activity of osteoclast; Bone trabecula forming surface percent (TFS%): the percent of osteoid surface with osteoblast incasement accounting for bone trabecula surface, which can judge osteoblast activity.

Detection of Expression of Wnt1, LRP-5 and ß-Catenin Proteins

Immunohistochemical staining was carried out according to the directions of the kits. After placed till room temperature, the freezing sections were immerged into 3% H_2O_2 for 30 min at the room temperature for inactivation of endogenous peroxidase; closed with sheep serum for 30 min; the antibody I (rabbit IgG) of 1: 200 dilution was dripped for overnight at 4 ℃, and 0.01M PBS replacing the antibody I was used the negative control; Then sheep-anti-rabbit two-step method antibody II (1:1 dilution) was dripped, 37 ℃, 30 min; DAB coloration kit was used for coloration. Campeachy was used for counter staining, followed by dehydration, hyalinization, mounting, and observation with microscope. Judgment of results: Cytoplasm of positive reactive cells of osteoporosis (OB) and bone marrow stromal cells (BMC) showed pale brown. Morphological measure analysis: Over 5 high power

fields (×400) in the marrow cavity below the epiphyseal plate were randomly detected with Leica-Qwin image analysis system, and positive area ratio of each visual field and the mean were calculated. The positive area ratio was percent of the positive cell area with pale brown staining in the cytoplasm in each visual field accounting for the area of bone trabecula within the visual field, which was used for positive reactive cell density (positive density for short).

Statistical Analysis

The data were expressed as mean ± standard deviation ($\bar{x} \pm s$), Differences between groups were assessed by a one-way analysis of variance (ANOVA) using SPSS11.0 software. Inter-group comparisons were performed using the least significant difference (LSD) test. Statistical significance was accepted at $P<0.05$.

RESULTS

Effects of Zuo Gui Pill on Bone Histomorphologic Indexes in the Rats of Glucocorticoid-induced Osteoporosis

TBV% of the tibia in the model group was significantly lower than that in the blank control group, while in the Zuo Gui Pill group was significantly higher than that in the model group, with a significant difference between the blank control group and the Zuo Gui Pill group. Compared with the blank control group, the tibial TRS% significantly increased and TFS% significantly decreased in the model group and the Zuo Gui Pill group; Compared with the model group, TRS% did not have significant change, but TFS% significantly increased (Table1).

Effects of Kidney-*yin*-nourishing Method on Expression of OB and MSC Wnt1 Proteins in the Tibia of the Rat with Glucocorticoid-induced Osteoporosis

Compared with the blank control group the Wnt l protein expression positive density of OB and MSC in the rat tibia in the model group was significantly decreased; and compared with the model group, it significantly increased in the Zuo Gui Pill group (Table 2).

Table 1. TBV%, TRS% and TFS% of the tibia of the rat in the groups ($\bar{x} \pm s$)

Group	Cases	TBV%	TRS%	TFS%
Blank control	12	32.31±4.75	3.63±0.91	7.72±2.10
Model	15	14.05±3.52**	7.05±1.59**	3.35±1.01**
Zuo Gui Pill	15	21.27±4.11$^{**\triangle\triangle}$	5.95±1.52**	6.03±1.47$^{*\triangle\triangle}$

Notes: Compared with the blank control group, *$P<0.05$, **$P<0.01$; Compared with the model group, $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$; The same bellow.

Table 2. Positive densities of Wnt 1 protein expression in OB and MSC in the tibia bone of the rat in the groups ($\bar{x} \pm s$)

Group	Cases	OB and MSC Wnt1 protein positive expression density
Blank control	12	1.90±0.34
Model	15	0.77±0.19**
Zuo Gui Pill	15	1.18±0.26$^{**\triangle\triangle}$

Effects of Kidney-*yin*-nourishing Method on Expression of OB and MSC LRP-5 Protein in the Tibia of the Rat with Glucocorticoid-induced Osteoporosis

Compared with the blank control group, the LRP-5 protein expression positive density of OB and MSC in the rat tibia in the model group significantly decreased; and compared with the model group, it significantly increased in the Zuo Gui Pill group with lower than that in the blank control group (Table 3).

Table 3. Positive densities of LRP-1 protein expression of OB and MSC in the tibia bone of the rat in the groups ($\bar{x} \pm s$)

Group	Cases	OB and MSC LRP-5 protein positive density
Blank control	12	2.13±0.35
Model	15	0.96±0.20**
Zuo Gui Pill	15	1.34±0.22$^{**\triangle\triangle}$

Effects of Kidney-*yin*-nourishing Method on Expression of OB and MSC ß-Catenin Proteins in the Tibia in the Rat with Glucocorticoid-induced Osteoporosis

Compared with the blank control group the ß-catenin protein expression positive density of OB and MSC in the rat tibia in the model group significantly decreased; and compared with the model group, it significantly increased in the Zuo Gui Pill group, but with lower than that in the blank control group (Table 4).

Table 4. Positive densities of ß-catenin protein expression of OB and MSC in the tibia of the rat in the groups ($\bar{x} \pm s$)

Groups	Cases	OB and MSC ß-catenin protein positive expression density
Blank control	12	2.09±0.30
Model	15	0.87±0.23**
Zuo Gui Pill	15	1.26±0.29$^{**\triangle}$

DISCUSSION

TCM holds that the essence of life stores in kidney dominates the bones and produces bone marrow. If the kidney-essence is sufficient, formation of bone marrow will have substance resource, and the bones will be nourished, hence very hard and forceful bones; if the kidney-essence is insufficient, formation of the marrow will lack substance resource, leading to fragile bones, pain in loin and back , lassitude in tibias and knees, etc.

In recent years, it has been found that Wnt signal transduction is a key pathway of cell development and regulating growth, and it plays an important role in embryonic development, and formation and functions of a part of adult tissues in normal situation. Many experiments prove that Wnt can directly influence the course of differentiation from multi-potent precursor cells to osteoblast. Stable expression of Wnt1, Wnt3α can promote proliferation of C3H10T1/2 cell line and induce activity of ALP, a early marker of osteoblast differentiation. In addition, Wnt1 and Wnt10b can inhibit differentiation of fat cells or pre-adipose cells, and in C3H10T1/2 over expression Wnt3α can inhibit expression of fat cell marker PPARγ2 and promote its differentiation towards osteoblast.[2] LRP5 exits on surface of osteoblast and has very important function in accumulation of bone quantity. Lost changes of its function can lead osteoporosis, and acquired change of the function can cause high bone quantity. LRP5 as trans-membrane receptor of Wnt proteins regulates proliferation and differentiation and other functions of osteoblast via Wnt /LRP5 signal transduction pathway to influence accumulation of human bone quantity. The newest study indicates that expression changes and function loss of LRP5 can lead to reduction of in vitro cultured rat skull bone density, with the bone substance thinned; in human development, it can influence acquisition of bone substances.[3,4] While ß-catenin plays role in osteogenic pre-cells and proliferation and differentiation of osteoblast.[5] It can be seen that Wnt proteins and trans-membrane proteins---LRP-5 and ß-catenin proteins are a key link of Wnt signal transduction pathway, and in recent years, their functions in bone metabolism are being drawn attention day by day.

Zuo Gui Pill is a represent prescription for nourishing *yin* and reinforcing kidney method and has a certain therapeutic effect on osteoporosis induced by "insufficient kidney-essence unable nourishing the bones". The research group observed therapeutic effects of Zuo Gui Pill in the postmenopausal osteoporosis model rat with ovariectomy and the results indicated that Zuo Gui Pill could regulate transmission of coupling signals between osteoblast and ostoclast,[6-9] and could regulate differentiation of osteoclast through regulating the expression ratio of OPG/RANKL protein and mRNA,[10,11] so as to cure osteoporosis induced by ovariectomy in the at. While the experiment observed its therapeutic effects on glucocorticoid-induced osteo-porosis in the rat and the results indicated that after a large quantity of glucocorticoid in succession, tibial TBV%, a main marker of bone amount, significantly decreased, and TRS% which represents bone absorption parameter significantly increased, and TFS% which represents bone forming parameter significantly decreased, indicating that induced by glucocorticoid is a osteoporosis model with decrease of bone formation, increase of bone absorption, and the bone absorption increase over the bone formation. After administration of Zuo Gui Pill, TBV% and TFS% significantly increased, TRS% did not change, suggesting that Zuo Gui Pill can control and reverse the bone loss due to increase of bone absorption and decrease of bone formation induced by glucocorticoid, leading to increase of bone amount.

In the study, effects of Zuo Gui Pill on the expression levels of Wnt1, LRP-5 and ß-catenin proteins in tibial osteoblast (OB) and bone marrow stromal cells (MBC) were investigated with immunohistochemical staining in the rat with osteoporosis induced by glucocorticoid, and the results indicated that the expression levels of Wnt1, LRP-5 and ß-catenin all were down-regulated; after administration of Zuo Gui Pill, the expression levels of Wnt1, LRP-5 and ß-catenin all were up-regulated, indicating that therapeutic effects of Zuo Gui Pill on glucocorticoid-induced osteoporosis are carried out through regulating expression of the key signal molecules of Wnt signal transduction pathway — Wnt1, LRP-5 and ß-catenin, possibly strengthening Wnt signals, increasing its combination with receptor LRP-5, thus ß-catenin protein can not be degraded, so as to aggregate in cells and enter the nucleus and combines with transcriptption factor TCF to activate the gene transcription course, thus, on the one hand, promoting orientation, differentiation, development, proliferation of osteoblasts, and inhibiting apoptosis of osteoblast; and on the other hand, inhibiting activity of osteoclasts through induction of osteoblasts, finally, making increase of bone formation and decrease of bone absorption, thus bone loss is controlled and restored.

In brief, the results showed that Zuo Gui Pill, a represent recipe for nourishing yin and reinforcing kidney method, has obvious therapeutic effects on glucocorticord-induced osteoporosis in the rat, which were carried out through up-regulation of expression levels of the key molecules of Wnt signal transduction pathway — Wnt1, LRP-5 and ß-catenin. On the one hand, it can promote orientation, differentiation, development, proliferation of osteoblasts, and inhibiting it apoptosis; and on the other hand, it can inhibit activity of osteoclasts through induction of osteoblasts, finally, increasing bone formation and decreasing bone absorption, hence control and recovery of bone amount lost state. It is indicated that Wnt signal transduction pathway plays very important role in "kidney dominating the bones" course. When Wnt signal pathway transduction is normal, growth, development and function of osteoblasts are at normal state, promoting bone formation, this is a manifestation of "sufficiency of bone marrow" stated in TCM; When Wnt signal transduction pathway is abnormal, the growth, development, and function of osteoblasts are inhibited, leading to decrease of bone formation, which is a manifestation of "insufficiency of bone marrow".

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