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Rosuvastatin promotes osteogenic differentiation of mesenchymal stem cells in the rat model of osteoporosis by the Wnt/β-catenin signal

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Abstract. – OBJECTIVE: The aim of this study was to explore the promoting effect of rosuvastatin on the osteogenic differentiation of mesenchymal stem cells in the rat model of osteoporosis through the Wnt/β-catenin signal.

MATERIALS AND METHODS: A total of 30 rats were purchased from the Animal Research Center of Shanxi Medical University. All rats were randomly allocated into three groups, including: group A (control group, n=10), group B (ovariectomized group, n=10), and group C (rosuvastatin gavage group, n=10). The bone metabolism indexes, bone mineral density (BMD) and the Wnt/β-catenin signaling pathway-related proteins in blood samples of rats in each group were measured, respectively. Furthermore, the bone marrow mesenchymal stem cells of rats were used for alkaline phosphatase (ALP) staining. All data were analyzed using the Statistical Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA).

RESULTS: The rats firstly received 9 consecutive weeks of feeding with drug intervention. The imaging results revealed that trabecular thickness in group A was significantly higher than that of group B and group C, showing statistically significant differences ($p<0.05$). After 9 consecutive weeks of feeding with drug intervention, BMD of the femurs of rats in group A and group C was significantly higher than that of group B, showing statistically significant differences ($p<0.05$). However, there was no significant difference in BMD between group A and group C ($p>0.05$). The level of calcium representing bone absorption level in serum of rats in group B was remarkably higher than that of group A, and the difference was statistically significant ($p<0.05$). However, the level of ALP representing bone absorption level in the serum of rats in group B was significantly lower than that of group A ($p<0.05$). No significant differences were found in the levels of calcium and ALP that represented bone absorption level between group C and group A ($p>0.05$). Meanwhile. the levels of phosphorus in the three groups were similar, showing no statistically significant difference ($p>0.05$). Moreover, the expression of ALP-positive cells in the rats of group A and group C was markedly higher than that of group B ($p<0.05$). After drug intervention through feeding for 9 consecutive weeks, no evident difference was found in the relative expression of Wnt/β-catenin signaling pathway-related protein glycogen synthase kinase-3β (GSK-3β) among the three groups. The relative expression of the protein phosphorylated GSK-3β (p-GSK-3β) in group C was significantly lower than that of group B ($p<0.05$). Furthermore, the relative protein expressions of β-catenin and cyclin D1 in group C were significantly higher than those in group B ($p<0.05$).

CONCLUSIONS: Rosuvastatin can improve bone metabolism in osteoporosis rats and increasing BMD of bone tissues in rats with osteoporosis. Besides, the Wnt/β-catenin signaling pathway plays a crucial role in the regulation of the stem cell self-renewal and bone genesis.

Key Words:
Rosuvastatin, Wnt/β-catenin, Osteoporosis, Osteogenic differentiation.

Introduction

The prevention and treatment of postmenopausal osteoporosis have always been a difficult issue in the scientific community. The treatment with different drugs are accompanied by side effects. For instance, estrogen supplementation slows down the rate of bone loss in postmenopausal women to a certain extent. However, the regular estrogen supplementation will increase the burden on women's liver and kidney. Meanwhile, this may greatly boost the incidence rates of uterine cancer, ovarian cancer, and breast cancer[1]. Statins are considered as the most potential

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drugs to promote bone formation[2]. Wallace et al[3] have pointed out that rosuvastatin exerts a promoting effect on bone formation. They have also found that rosuvastatin effectively activates osteoblasts and stimulates the self-renewal of mesenchymal stem cells. Wnt proteins regulate the proliferation and differentiation of osteoblasts through different mechanisms. It has been demonstrated[4,5] that its pathway plays a key role in the differentiation process of various bone tissues. In addition, Wnt proteins induce osteoblast generation and modulate osteocyte differentiation, which is closely associated with osteoporosis, arthritis, femoral head necrosis, and other diseases[6]. However, researches have elucidated the effects of rosuvastatin and Wnt/β-catenin signals on the osteogenic differentiation of osteoporotic stem cells in China.

In this study, a rat model of postmenopausal osteoporosis was successfully established by ovariectomy. The effect of rosuvastatin on the osteogenic differentiation of mesenchymal stem cells was explored. Furthermore, the role of the Wnt/β-catenin signaling pathway in promoting bone formation by rosuvastatin was further analyzed. Our findings might provide a certain theoretical basis for the biological research of bone health.

Materials and Methods

Experimental Animals and Grouping

A total of 30 rats (3-month-old, weighing 0.16-0.19 kg) were purchased from the Animal Research Center of Shanxi Medical University. All rats were kept under the conditions of 25°C, humidity of 45%, and a 12 h/12 h light/dark cycle. Meanwhile, they were given free access to food and water. After 1 day of adaption all rats were randomly allocated into three groups, including: group A (control group, n=10, fed normally without any treatment), group B (ovariectomized group, n=10, fed with normal feeding after ovariectomy) and group C (rosuvastatin gavage group, n=10, received rosuvastatin gavage at a dose of 20 $mg \cdot kg^{-1} \cdot d^{-1}$ for 9 consecutive weeks after ovariectomy). This study was approved by the Animal Ethics Committee of Liaocheng People's Hospital Animal Center.

Main Instruments and Reagents

The main instruments and reagents applied in this research were as follows: cryogenic centrifuge (Eppendorf, Hamburg, Germany), liquid scintillator (model: 1450, Wallac, Waltham, MA, USA), (BMD) apparatus (Lunar Prodigy, Boston, MA, USA), fluorescence quantitation kits (Toyobo, Osaka, Japan), alkaline phosphatase (ALP) detection kits (NGB Co., Ltd., Shanghai, China), goat anti-rabbit antibodies (Abcam, Cambridge, MA, USA), reverse transcription kits (QIAGEN, Hilden, Germany), and trypsin and culture media (Hyclone, South Logan, UT, USA).

Establishment of Osteoporosis Model in Rats

The rats were anesthetized by intraperitoneal injection of 1% pentobarbital sodium at a dose of 0.1 g per kg of body weight. The ovariectomy was then carried on both sides in rats of group B and group C. The specific methods were as follows: the long hairs on both sides of the lumbar spine were shaved first. A small opening was made on the left and right of the lumbar spine, respectively. After that, ovaries on both sides of rats were removed with scorching tweezers. The wounds were then sutured using catguts. Finally, a small quantity of drugs was smeared on the wound surface to diminish inflammation.

Collection of Specimens and Culture of Bone Marrow Mesenchymal Stem Cells

After 9 consecutive weeks of feeding with drug intervention, the rats in the three groups were anesthetized by intraperitoneal injection of 1% pentobarbital sodium at a dose of 0.1 g per kg of body weight. After the rats were killed, the bilateral femurs were taken out under aseptic condition and refrigerated for storage for subsequent use. After that, BMD (femurs stored at -20°C) and Wnt/β-catenin signaling pathway-related proteins (femurs stored at -80°C) were detected. The blood samples were then collected from the abdominal aortae of rats. The levels of bone metabolism indexes, ALP, calcium, and phosphorus were measured, respectively. The isolation and culture of mouse bone marrow mesenchymal stem cells were conducted according to the methods in literature[7]. Subsequently, pre-cooled Phosphate-Buffered Saline (PBS) was used to wash femurs to obtain bone marrow cells, followed by centrifugation at 300 × g and room temperature for 5 min. The mesenchymal stem cells were cultured in DMEM/F12 complete medium containing 150 g/L FBS. The medium was

replaced every 2-3 d. In this study, the mouse bone marrow mesenchymal stem cells that were passaged to 3-5 generations were used.

ALP Staining

The mouse bone marrow mesenchymal stem cells were stained according to the instructions of the ALP detection kit. The cytoplasm and nuclei of ALP-positive cells turned black, blue, and reddish brown, respectively.

Determination of BMD of the Femurs

The frozen femurs were first thawed at room temperature. The BMD of the femurs in rats was directly measured using the BMD apparatus.

Determination of Proteins Related to the Wnt/β-Catenin Signaling Pathway

The femurs were first taken out, chopped up and ground into powder after the addition of liquid nitrogen. After adding the tissue lysate containing the protease inhibitors, grinding was performed for 15 min. After that, the relative expression level of proteins associated with the Wnt/β-catenin signaling pathway was measured according to the method in literature[8]. The average density of each protein was expressed as the ratio of cyclin D1/β-actin, phosphorylated glycogen synthase kinase-3β (p-GSK-3β)/β-actin, β-catenin/β-actin, and GSK-3β/β-actin, respectively.

Detection of mRNA Expression via Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The messenger ribonucleic acid (mRNA) expression level of genes was detected as follows. The total RNA was extracted from osteoblasts according to the instructions of the TRIzol reagent. 1 μg extracted RNA was synthesized into complementary deoxyribose nucleic acid (cDNA) in strict accordance with reverse transcription kit. Subsequently, 1 μL forward and 1 μL reverse amplification primers with a concentration of 10 μmol/L were added, respectively. 10 μL SSO-Fast™ EVA Green Mix (Bio-Rad, Hercules, CA, USA) was placed on a quantitative PCR instrument based on a 2-fold reaction system. Finally, the mRNA expression of target genes was measured (Table I).

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA) was used for all statistical analyses. The BMD, ALP, calcium, and phosphorus of rats in group A, group B, and group C were compared using the *t*-test. The univariate analysis was performed to compare the differences among different groups. The count data were expressed as ($\bar{x} \pm s$). $p<0.05$ was considered statistically significant.

Results

Imaging Test Results

After rats in the three groups received 9 consecutive weeks of feeding with drug intervention, then, the imaging test was carried out. The results showed that the trabecular thickness in rats of group A was significantly higher than that of group B and group C ($p<0.05$) (Figure 1).

Comparison of BMD in the Femurs of Rats

The BMD in the femurs of rats in group A and group C was higher than that of group B after 9 consecutive weeks of feeding with drug intervention, and the differences were statistically significant ($p<0.05$). However, no evident difference was found in BMD between group A and group C ($p>0.05$) (Figure 2).

Table I. Gene primer sequences.

Gene	Primer sequence
β-catenin	Forward: 5'ATGGAGCCGGACAGAAAAGC3' Reverse: 5'CTTGCCACTCAGGGAAGGA3'
β-actin	Forward: 5'CAGAGCCTCGCCTTTGCCGATC3' Reverse: 5'GGCCTCGTCGCCCACATAGG3'
GSK-3β	Forward: 5'GCACCACCGTCAGCAACA3' Reverse: 5'CCTGGCATCGGCAAACTC3'
Cyclin D1	Forward: 5'GGTGGCAAGAGTGTGGAG3' Reverse: 5'CCTGGAAGTCAACGGTAGC3'
P-Gsk-3β	Forward: 5'GCGGGTGCAGCTTGAAAATC3' Reverse: 5'GCACTGCTCACCTTCACGA3'

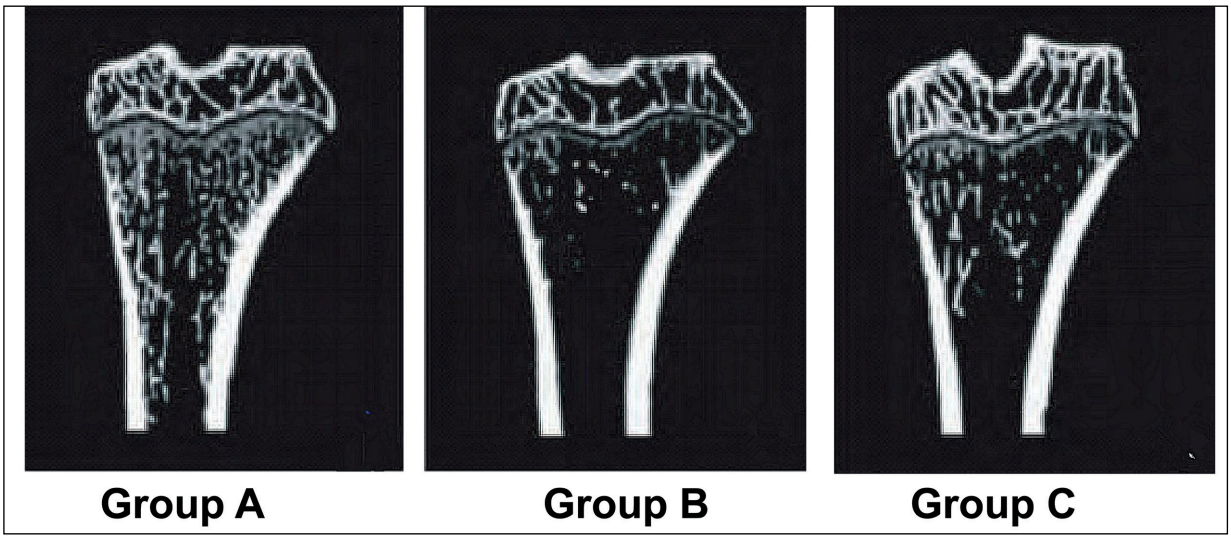

Figure 1. Imaging test after 9 consecutive weeks of feeding with drug intervention.

Comparisons of Bone Metabolism Indexes in Rat Serum

The level of calcium representing bone absorption level in the serum of rats in group B was significantly higher than that of group A ($p<0.05$). However, the ALP level that represented bone absorption level in the serum of rats in group B was lower than that of group A, showing statistically significant differences ($p<0.05$). No significant differences were found in the levels of calcium and ALP that represented the bone absorption level between group C and group A ($p>0.05$). Meanwhile, the levels of phosphorus in the three groups were similar, showing no statistically significant difference ($p>0.05$) (Table II).

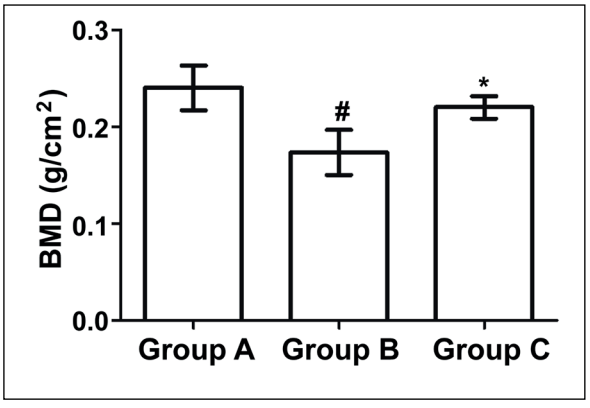

Figure 2. Comparison of BMD in the femurs of rats in the three groups. Note: $^{\#}p<0.05$ *vs.* group A, and $^{*}p<0.05$ *vs.* group B.

ALP Staining

The expression level of ALP-positive cells in rats of group A and group C was markedly higher than that of group B after 9 consecutive weeks of feeding with drug intervention ($p<0.05$) (Figure 3).

Expression Levels of Proteins Associated With Wnt/β-Catenin Signaling Pathway in Bone Tissues of Rats

After the rats underwent 9 consecutive weeks of feeding with drug intervention, the Wnt and β-catenin proteins were detected as shown in Figure 4. The results revealed that no significant difference was observed in the relative expression level of Wnt/β-catenin signaling pathway-related protein GSK-3β among the three groups. However, the relative protein expression level of p-GSK-3β in group C was lower than that of group B, displaying statistically significant differences ($p<0.05$). However, the relative protein expression levels of β-catenin and cyclin D1 in group C were significantly higher than those of group B ($p<0.05$) (Figure 5).

Discussion

Osteoporosis is a major cause of bone pain and bone injury in the middle-aged and elderly population. After menopause, women are prone to fracture due to bone mass reduction, bone tissue metabolism changes, and BMD reduction[9]. With

Table II. Comparisons of bone metabolism indexes in rat serum.

	Group A	Group B	Group C	*F*	*p*
ALP (U/L)	236.52 ± 13.11	187.34 ± 13.4#	234.85 ± 18.29*	5.831	< 0.001
Calcium (mmol/L)	2.14 ± 0.17	2.53 ± 0.08#	2.28 ± 0.15*	2.049	0.043
Phosphorus (mmol/L)	2.25 ± 0.23	2.36 ± 0.07	2.26 ± 0.17	0.48	0.632

Note: #$p<0.05$ *vs*. group A, and *$p<0.05$ *vs*. group B.

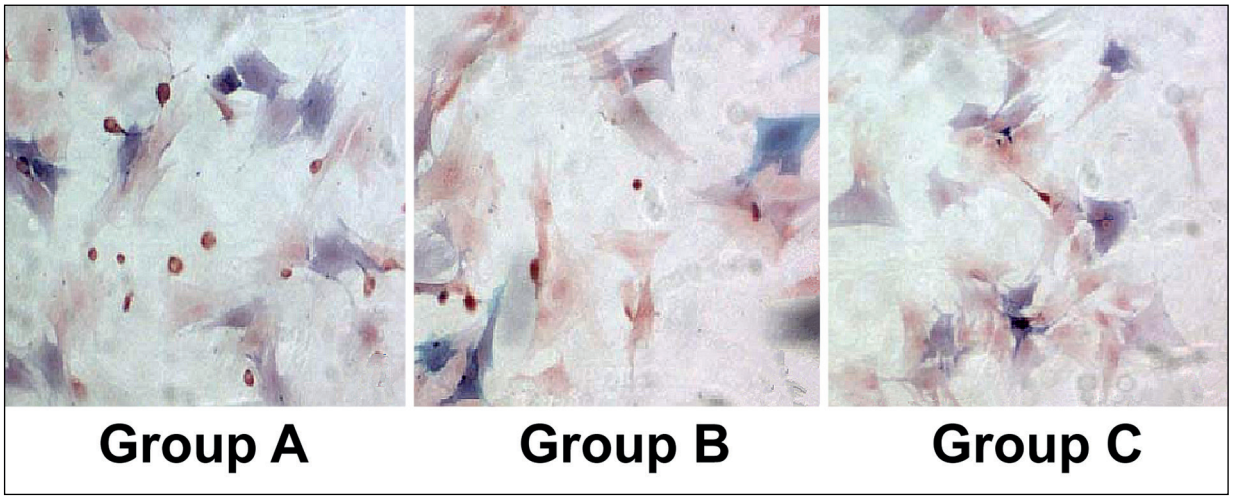

Figure 3. ALP staining in rats. Note: black blue area is the cytoplasm of ALP-positive cells. Cytoplasm of ALP-positive cells in the rats in 3 different groups (magnification: 100×).

the aggravation of global aging, the osteoporosis has become a relatively serious problem in the world, severely affecting the physical and mental health of the elderly. In this study, evident osteoporosis symptoms appeared after the removal of bilateral ovaries in rats. These were manifested as remarkably lower BMD in group B than that of group A. The above results indicated that the modeling method of osteoporosis induced by ovariectomy was effective. Meanwhile, a reduced BMD of the modeling rats was correlated with the rapid decrease in the estrogens. This result was similar to the occurrence and development processes of postmenopausal osteoporosis in women[10,11]. According to the results of this study, the BMD of rats in group C and group B was significantly higher than that of group B after 9 consecutive weeks of feeding with drug intervention. These findings suggested that rosuvastatin could notably increase the BMD of rats through the Wnt/β-catenin signals.

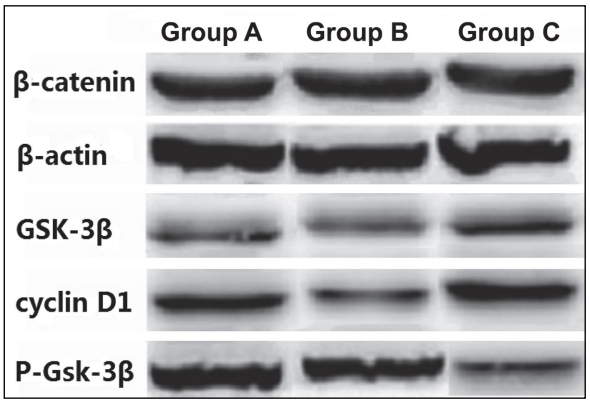

Figure 4. Expressions of Wnt/β-catenin signaling pathway-related proteins in rats of the three groups.

After the rats received 9 consecutive weeks of feeding with drug intervention, the calcium level representing bone absorption in rat serum in group B was significantly higher than that of group A ($p<0.05$). However, the ALP level that represented bone absorption in the rat serum of group B was remarkably lower than that of group A ($p<0.05$). In addition, the levels of calcium and ALP representing bone absorption in the rat serum of group C were similar to those of group

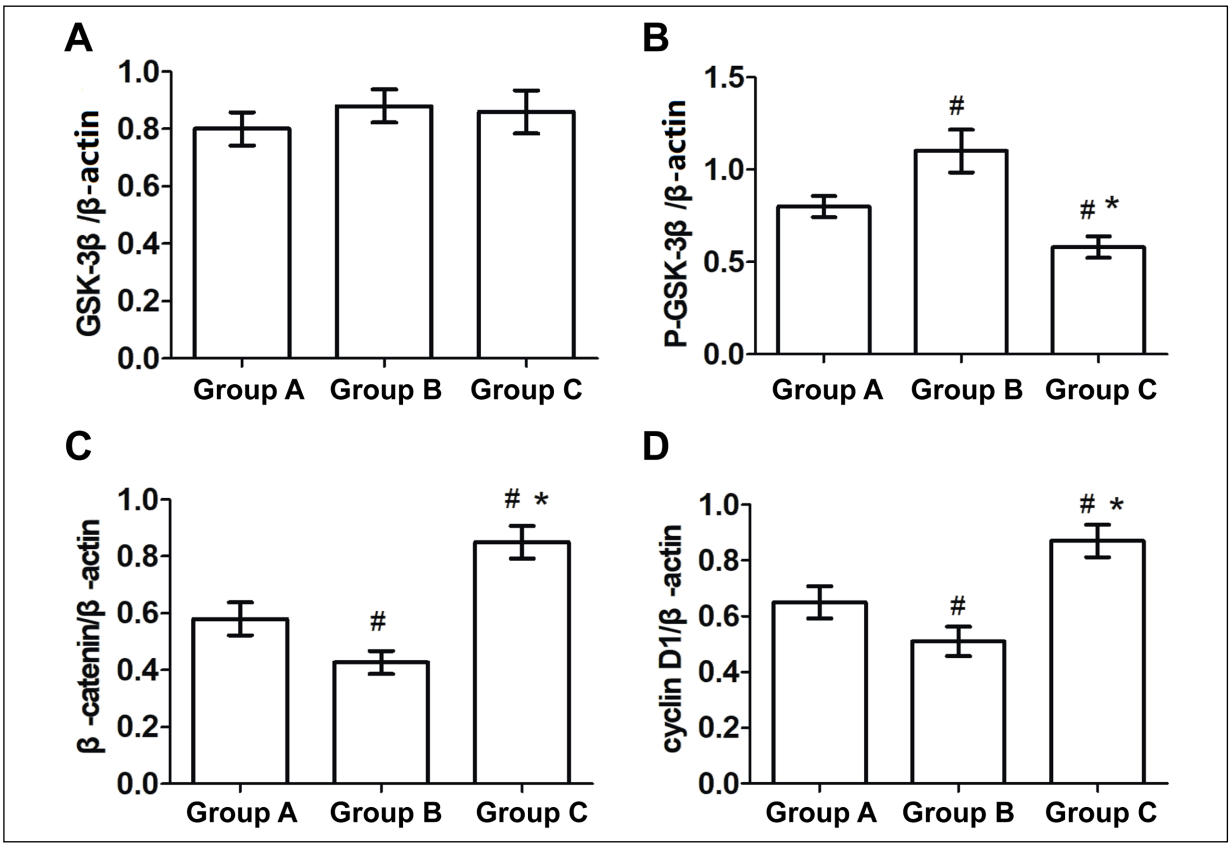

Figure 5. Differences in the expression level of Wnt/β-catenin signaling pathway-related proteins in the rats of the three groups. Note: $^{\#}p<0.05$ *vs.* group A, and $^{*}p<0.05$ *vs.* group B. **A,** The relative expression level of protein GSK-3β. **B,** The relative expression level of protein p-GSK-3β. **C,** The relative expression level of protein β-catenin. **D,** The relative expression level of protein cyclin D1. β-actin was used as a control.

A. Meanwhile, the phosphorus levels in rat serum in the three groups were similar, showing no significant differences ($p>0.05$). Chen et al[12] proved that calcium, phosphorus, and ALP are biochemical markers of bone metabolism in serum and this is well kown. Elevated serum calcium often indicates accelerated dissolution of bones, representing a bone loss. There is a certain proportion between serum calcium and phosphorus. Meanwhile, the concentration of serum calcium and phosphorus can reflect the degree of bone absorption to a certain extent. ALP, as a biochemical marker of bone formation, effectively reflects the activity of osteoblasts[13]. Zain et al[14] have found that statins can evidently improve the mRNA expression levels of Coil, ALP, OCN, and OPN in mice, increase BMD and bone strength, and promote new bone formation, which is similar to the results of our study[15].

After the rats underwent 9 consecutive weeks of feeding with drug intervention, no significant difference was found in the relative expression level of Wnt/β-catenin signaling pathway-related protein GSK-3β among the three groups. However, the relative protein expression level of p-GSK-3β in group C was lower than that of group B, displaying statistically significant differences ($p<0.05$). The relative protein expression levels of β-catenin and cyclin D1 in group C were remarkably higher than those of group B ($p<0.05$). Wnt/β-catenin signaling pathway, one of the most classical signaling pathways, plays a vital role in the differentiation of varying bone tissues[16]. As the most pivotal nuclear transcription factor in the Wnt/β-catenin signaling pathway, cyclin D1 modulates cell cycle, regulates osteoblast proliferation, and stimulates bone formation[17-19]. When the Wnt protein is not activated, GSK-3β can phosphorylate β-catenin and further degrade β-catenin[17,18]. Nevertheless, the activated Wnt protein is able to inhibit the phosphorylation process of β-catenin by GSK-3β, further enabling β-catenin to accumulate them inside the nucleus. Scholars[19,20] have also demonstrated that β-cat-

enin plays its role in regulating the osteoblast proliferation by activating the transcription of its downstream gene protein cyclin D1. Rosuvastatin suppresses the expression of p-GSK-3β through Wnt/β-catenin signals, so as to reduce the phosphorylation of β-catenin by p-GSK-3β. In addition, rosuvastatin can reduce the degradation of β-catenin, increase the accumulation of β-catenin in cells, and promote the proliferation of osteoblasts by activating its downstream gene protein cyclin D1[21-23].

Conclusions

Rosuvastatin is capable of improving bone metabolism and BMD in osteoporosis rats. Wnt/β-catenin signaling pathway exerts crucial regulatory effects on stem cell self-renewal and bone genesis. Moreover, rosuvastatin can effectively stimulate the osteogenic differentiation of bone marrow mesenchymal stem cells in rats with osteoporosis through the Wnt/β-catenin signals.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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