Effects of Epimedium Extract on Osteoporotic Fracture Healing in Rats via the Notch Signaling Pathway

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ABSTRACT

Objective

To evaluate the effects of Epimedium extract on osteoporotic fracture healing in rats via the Notch signaling pathway.

Methods

A total of 60 female Sprague-Dawley rats were randomly divided into sham operation group (Sham group, n=12), osteoporotic fracture model group (OVX group, n=12), low-dose icariin group (ICA-L group, n=12), medium-dose icariin group (ICA-M group, n=12) and high-dose icariin group (ICA-H group, n=12). Ovariectomy was performed in each group except Sham group, and the rat model of femoral fracture was established after 3 months. After successful modeling, the rats in Sham group and OVX group were gavaged with 0.9% sodium chloride solution at a dose of 10 mL/(kg·d-1), while those in ICA-L, ICA-M and ICA-H groups were gavaged with ICA solution at a dose of 50 mg/(kg·d-1), 100 mg/(kg·d-1) and 150 mg/(kg·d-1), respectively. After drug administration for 12 consecutive weeks, the bone mineral density (BMD) of the femur was detected using a dual-energy X-ray bone densitometer, the content of serum osteocalcin (OC), tartrate-resistant acid phosphatase (TRAP), alkaline phosphatase (ALP) and estradiol (E2) was determined using kits, and the bone trabecula and changes in trabecular microstructure were detected through micro-CT scan. The protein expression levels of Notch1, Jagged1 and Hes-1 in bone tissues were determined using Western blotting.

Results

After drug administration for 12 weeks, the content of serum ALP and TRAP significantly declined, while the content of E2 and OC rose in ICA groups compared with those in OVX group, and they had statistically significant differences between ICA-H group and OVX group ($P<0.05$). In ICA groups,

the trabecular number (Tb.N), trabecular thickness (Tb.Th), bone volume/tissue volume ratio (BV/TV) and BMD were increased compared with those in OVX group, showing statistically significant differences ($P<0.05$), while the trabecular separation (Tb.Sp) was significantly smaller than that in OVX group, showing a statistically significant difference ($P<0.05$). Western blotting revealed that ICA obviously increased the protein expressions of Notch1, Jagged1 and Hes-1 in femoral tissues of rats.

Conclusion

Epimedium extract ICA exerts a good therapeutic effect on rats with osteoporotic fractures, whose mechanism is that ICA upregulates Notch1, Jagged1 and Hes-1 protein expressions, thereby regulating the Notch signal transduction pathway.

INTRODUCTION

Osteoporosis (OP) is a metabolic bone disease caused by the imbalance between bone resorption and bone formation, manifested as osteopenia, bone microstructure degradation and decline in bone strength,[1,2] thereby increasing the bone fragility and risk of fractures in patients. With the aging of the global population, the number of people suffering from OP and osteopenia continuously increases,[3] and the mortality rate of diseases caused by osteoporotic fractures has exceeded the sum of that of the three major gynecological tumors (breast cancer, cervical cancer, and corpus carcinoma), and also shows an increasing trend annually,[4] seriously threatening public health. Clinically, OP can be divided into primary OP and secondary OP. Primary OP is more common in postmenopausal women, while secondary OP can occur in kidney disease, endocrine disease, and connective tissue disease. OP is mainly treated with drug intervention, including bone resorption inhibitors (such as estrogen agonists and calcitonin) and bone formation inhibitors (e.g., thyroid hormone, vitamin D and its derivatives).[5] However, adverse reactions may occur during treatment, or the long-term compliance is poor.[6,7] Epimedium has a curative effect on OP and fractures, and it is one of the most commonly used traditional Chinese medicines for OP. 7 The main active ingredient of Epimedium is icariin (ICA). ICA can prevent and treat OP in rats caused by ovariectomy, tretinoin, and hormones,[[8] and it can also be stably and locally released while promoting bone formation and inhibiting bone resorption. Therefore, the Epimedium extract is rapidly becoming an alternative drug for the prevention and treatment of OP.[9]

The process of bone metabolism is regulated by many factors such as signal transduction pathways, transcriptional control, genetic factors, and hormones.[10] The Wnt/β-catenin, BMP/Smads, and OPG/RANKL signaling pathways play key roles in the process of bone metabolism. According to the latest study, the Notch signaling pathway, through mediating information conduction among osteocytes, can also regulate the proliferation and differentiation of osteoblasts and osteoclasts,[11] thereby playing a vital role in bone metabolism. However, whether ICA can regulate bone metabolism through the Notch signaling pathway is rarely studied.

In the present study, therefore, Sprague-Dawley (SD) rats were used as the objects of study, the rat model of osteoporotic fractures was established, and the protein expressions of Notch1, Jagged1, and Hes-1 in the Notch signaling pathway were determined, so as to explore the therapeutic effect of ICA on rats with osteoporotic fractures and the regulatory effect of Notch signaling pathway on bone metabolism in OP rats.

MATERIALS AND METHODS

Experimental Animals

A total of 60 3-month-old SPF female SD rats weighing (250±20) g were purchased from Beijing Vital River Laboratory Animal Co., Ltd. [license No.: SCXK (Beijing) 2012-0001]. Before the experiment, they were adaptively fed for 1 week under a temperature of 20-26°C, a relative humidity of 40-70% and normal diurnal cycle, and had free access to food and water.

Main Reagents and Apparatus

Epimedium extract (ICA content: 54.56%) was purchased from Changsha Yingrun Biotechnology Co., Ltd. Alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) kits were purchased from Nanjing Jiancheng Bioengineering Institute, and osteocalcin (OC), estradiol (E2) and bicinchoninic acid (BCA) protein assay kits were purchased from Nanjing KeyGEN Co., Ltd. Notch1, Jagged1 and Hes-1. Monoclonal antibodies and rabbit anti-rat GAPDH polyclonal antibody were bought from Cell Signaling Technology.

An XR-36 dual-energy X-ray bone densitometer was bought from NORLAND (USA), a multifunctional microplate reader was bought from TECAN (China), and a low-temperature high-speed centrifuge was bought from Jouan SA (France). A micro-CT system was provided by SCANCO Medical AG (Switzerland), and an inverted microscope was provided by Olympus (Japan).

Grouping and Modeling

After adaptive feeding for 1 week, the rats were randomly divided into sham operation group (Sham group, n=12) and ovariectomy group (n=48). The OP model was established via bilateral ovariectomy in all rats in ovariectomy group. In Sham group, only a few tissues around the ovary were removed, and the rats were intramuscularly injected with penicillin (200,000 U/d) for 3 consecutive days after operation. After normal feeding for 12 weeks, the bone mineral density (BMD) was detected using a dual-energy X-ray bone densitometer. After successful modeling, the rats in ovariectomy group were randomly divided into osteoporotic fracture model group (OVX group, n=12), low-dose icariin group (ICA-L group, n=12), medium-dose icariin group (ICA-M group, n=12), and high-dose icariin group (ICA-H group, n=12). The unilateral fracture model of the right proximal femur was established in all rats except Sham group [12], and the rats were intramuscularly injected with penicillin (200,000 U/d) for 7 consecutive days after operation.

Administration Methods

The rats in ICA-L, ICA-M, and ICA-H groups were gavaged with ICA solution at a dose of 50 mg/(kg·d-1), 100 mg/(kg·d-1) and 150 mg/(kg·d-1), respectively, for 12 weeks. In Sham group and OVX group, the rats were gavaged with an equal volume of 0.9% sodium chloride solution.

Measurement of Femoral BMD

After drug administration for 12 weeks, the rats were anesthetized with intraperitoneal injection of 3% pentobarbital sodium, and BMD of the left femur was measured using the dual-energy X-ray bone densitometer.

Detection of Bone Metabolism Indices

After drug administration for 12 weeks, the blood was drawn from the fundus venous plexus using a capillary glass tube. After standing for 2 h, the blood was centrifuged in a high-speed refrigerated centrifuge at 3,000 rpm for 15 min, and the serum was obtained and stored at -20°C for later use. After fasting for 24 h, the urine was collected and centrifuged at 3,000 rpm for 10 min, and the supernatant was harvested. Finally, the content of serum OC, TRAP, ALP, and E2 was determined using kits.

Micro-CT Scan

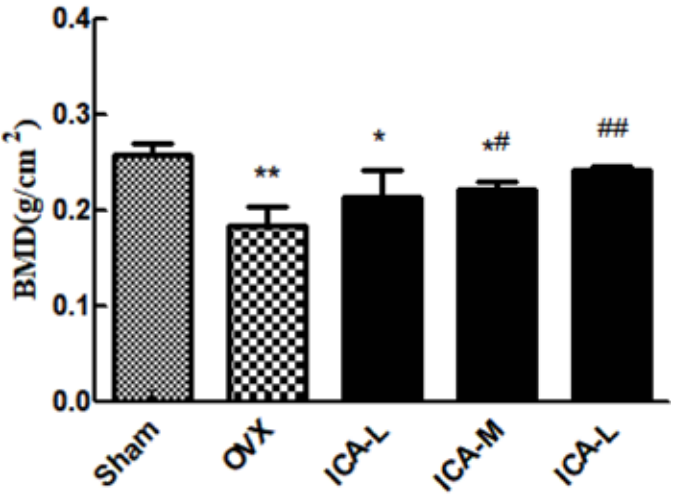
The femur was separated from LV4 and 5, from which the soft tissues were removed, and it was stored at -80°C for later analysis. The microstructure of the bone trabecula of the right proximal femur was analyzed through micro-CT (60 KV, 50 W). The same specimen was scanned to obtain different cross-sectional images, and the metaphysis of distal femur was subjected to three-dimensional scan. Then the following parameters were calculated using MicroView software (version 4.1; Scanco Medical AG, Wangen-Bruttisellen, Switzerland):

- bone volume/tissue volume ratio (BV/TV, %)
- trabecular number (Tb.N, mm-1)
- trabecular thickness (Tb.Th, mm)
- trabecular separation (Tb.Sp, mm).

Femoral Histological Assay

After deep anesthesia, the rats were sacri-

Figure 1. *BMD of each group. *P<0.05, **P<0.01 vs. Sham group; #P<0.05, ##P<0.01 vs. OVX group.*



ficed. The right femoral tissues were harvested in each group, decalcified with 20% EDTA for 3 weeks, chemically mounted with 4% formaldehyde for 48 h, dehydrated with gradient ethanol, washed with xylene, immersed in paraffin at 60°C overnight, and embedded in a paraffin block. Then the block was sliced into 5 μm-thick sections and stained with HE, and the pathological changes in femoral tissues were observed under a microscope.

Detection of Femoral Notch1, Jagged1 and Hes-1 Protein Expressions by Western Blot

After drug administration for 12 weeks, the rats were deeply anesthetized and sacrificed in each group. The left femur was harvested, the muscles and connective tissues attached to both ends and diaphysis of the femur were removed, and the femur was frozen with liquid nitrogen for 30 min and ground into powder. Then the powder was added with protein lysis buffer and centrifuged using the high-speed refrigerated centrifuge at 12,000 rpm for 15 min, and the supernatant was collected to obtain the protein. After SDS-PAGE, the protein was transferred onto a PVDF membrane, incubated with Notch1, Jagged1, Hes-1, and GAPDH primary antibodies at room temperature for 1 h, and then incubated again with horseradish peroxidase-labeled secondary antibodies at room temperature for 1 h, followed by color development using NCIP/NBT. The gray value of band was analyzed using gel imaging system.

Statistical Analysis

The experimental data were analyzed using SPSS 19.0 software. Graphpad Prism 5.0 software was used for plotting. The differences among groups were compared by one-way analysis of variance. P<0.05 was considered statistically significant.

RESULTS

Effects of ICA on BMD

After drug administration for 12 weeks, BMD of the femur was detected using the dual-energy X-ray bone densitometer in each group. The results showed that BMD

Figure 2. *Pathological staining sections of fractured end of femur. A) Sham group; B) OVX group; C) ICA-L group; D) ICA-M group; E) ICA-H group.*

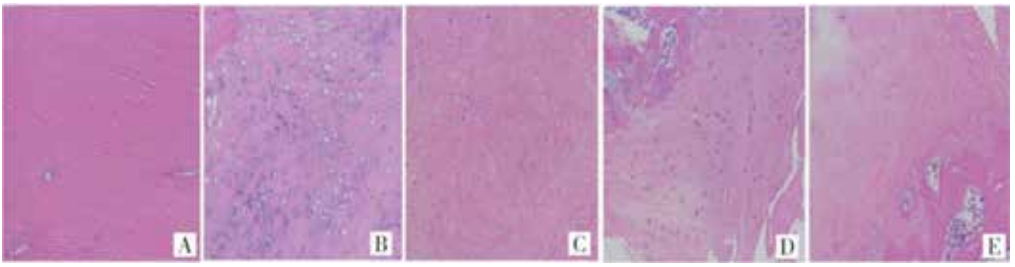
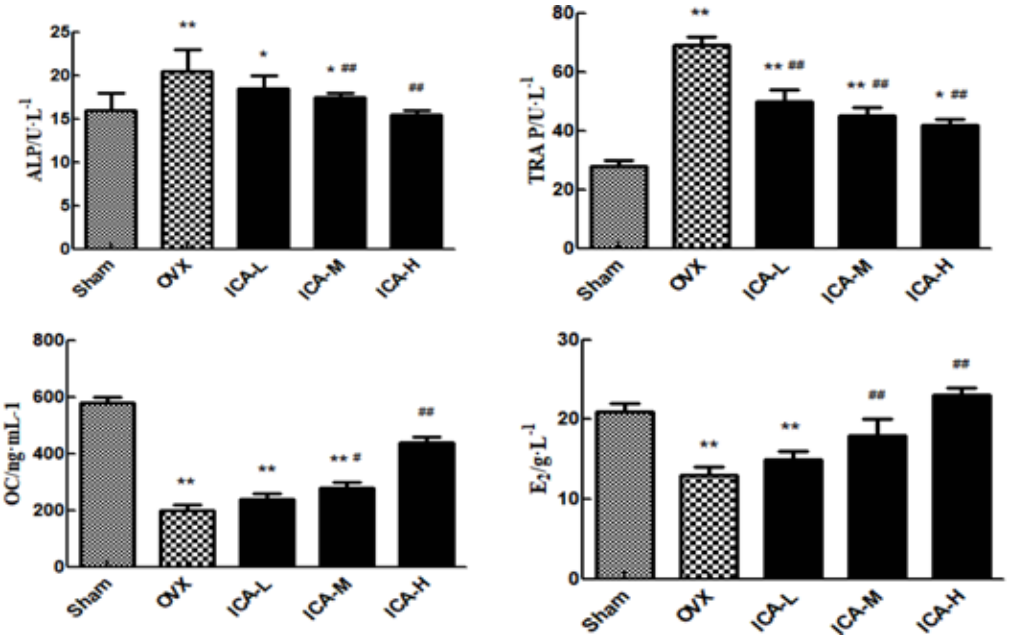


Figure 3. *Effects of ICA on serum ALP, TRAP, E2 and OC in rats with osteoporotic fractures. *P<0.05, **P<0.01 vs. Sham group; #P<0.05, ##P<0.01 vs. OVX group.*



significantly declined in OVX group compared with that in Sham group (P<0.01), while it rose in ICA groups compared with that in OVX group, showing statistically significant differences between ICA-M and ICA-H groups and OVX group (P<0.05, P<0.01) (Figure 1 and 2). Thus, ICA can increase BMD in rats with osteoporotic fractures.

Serum ALP, TRAP, E2 and OC Changes

After drug administration for 12 weeks, the content of serum ALP, TRAP, E2, and OC was detected using kits. The results revealed that OVX group had significantly increased content of serum ALP and TRAP, and significantly decreased content of E2 and OC compared with Sham group, displaying statistically significant differences (P<0.01). Besides, the content of serum ALP and TRAP declined, while the content of E2 and OC rose in ICA groups compared with those in OVX group, and they had statistically significant differences between ICA-M and ICA-H groups and OVX group (P<0.05) (Figure 3).

Figure 4. *Effect of ICA treatment on bone trabecula in rats with osteoporotic fractures.*

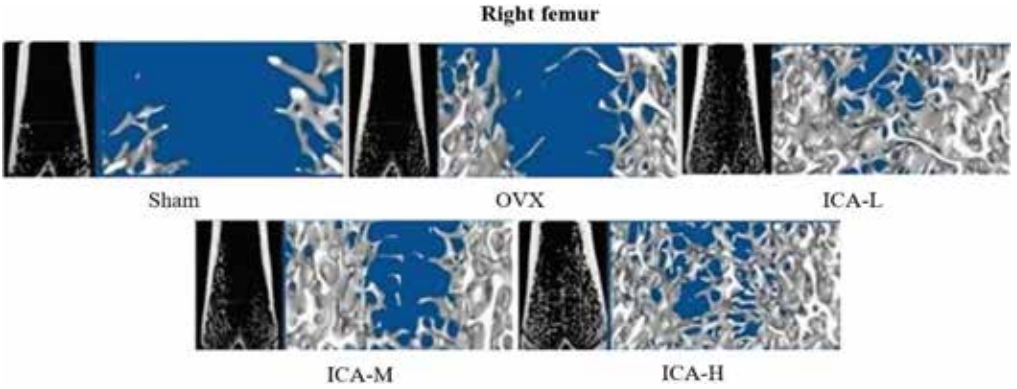
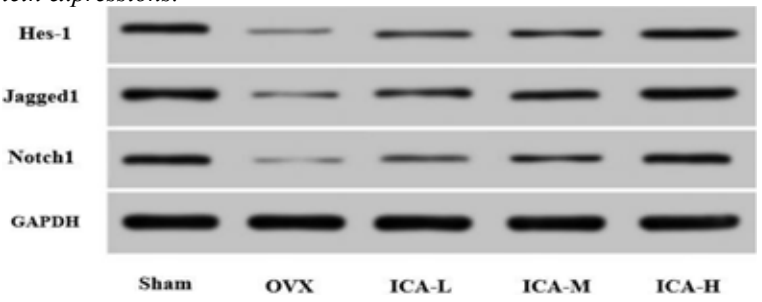


Figure 5. *Western blotting bands of Notch1, Jagged1 and Hes-1 protein expressions.*



Bone Trabecula Changes

It was observed through micro-CT that Tb.N was larger and Tb.Sp was smaller at the right distal femur in Sham group compared with those in OVX group. In OVX group, the bone trabecula was in a rod shape and became thinned and ruptured, and Tb.Sp was increased. ICA groups had larger Tb.N than OVX group, especially ICA-M and ICA-H groups. In ICA groups, Tb.Th was similar to that in Sham group, and some trabecular bones were lost despite decreased Tb.Sp. In ICA-L group, Tb.Sp was increased, but improved compared with that in OVX group (Figure 4).

Effect of ICA on Microstructure of Bone Trabecula

Compared with Sham group, OVX group had significantly decreased BV/TV, Tb.N, and Tb.Th, and significantly increased Tb.Sp of the femur, with statistically significant differences ($P<0.01$). BV/TV, Tb.N, Tb.Th, and Tb.Sp had no statistically significant differences between ICA-H groups and Sham group ($P>0.05$). Compared with those in OVX group, BV/TV, Tb.N, and Tb.Th were increased in ICA groups, showing statistically significant differences between ICA-M and ICA-H groups and OVX group ($P<0.01$), while Tb.Sp was significantly decreased ($P<0.05$, $P<0.01$) (Table 1).

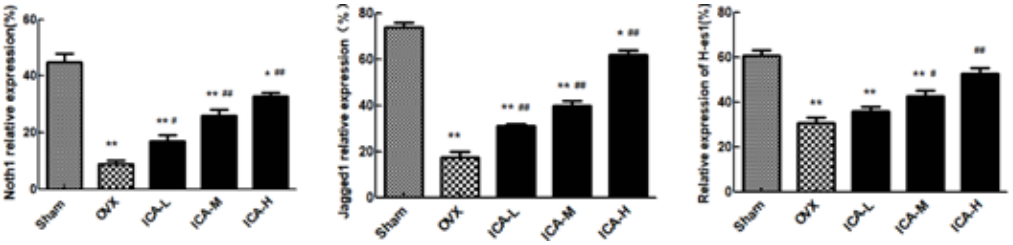
Effects of ICA on Notch1, Jagged1 and Hes-1 Protein Expressions

After drug administration for 12 weeks, the protein expressions in the femur were determined using Western blotting in each group. The protein expressions of Notch1, Jagged1, and Hes-1 significantly declined in OVX group compared with those in Sham group, with statistically significant differences ($P<0.05$), while they significantly rose in ICA groups compared with those in OVX group, with statistically significant differences ($P<0.05$) (Figure 5 and 6).

DISCUSSION

The main mechanism of osteoporotic fractures is that the balance between the osteogenesis of osteoblasts and the bone resorption of osteoclasts is broken, leading to osteopenia, decline in BMD, and increase in bone fragility. Fractures will occur when the load on the bone exceeds a certain limit, so anti-OP treatment should be the priority in the treatment of osteoporotic fractures. Drugs, exercise, and hormone replacement therapy are usually used in the prevention and treatment of OP.[13,14] Hormone replacement therapy is the most widely used, but it

Figure 6. *Western blotting results of Notch1, Jagged1 and Hes-1 protein expressions.*



has been confirmed that long-term treatment with these drugs may cause adverse reactions such as an increased risk of ovarian cancer and endometrial cancer. At present, it has been proved that many Chinese herbal medicine compound preparations exert significant effects in the clinical treatment of OP. Epimedium is a kind of common kidney-nourishing traditional Chinese medicine in clinical practice, with the main ingredient of ICA. It not only has an estrogen-like effect, but also can promote the proliferation of osteoblasts and inhibit the formation of osteoclasts.[15] The treatment of osteoporotic fractures with ICA has been reported in China and foreign countries. For example, Luo et al.16 showed that ICA played a role in restoring the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) in ovariectomized rats through the estrogen pathway.

Decline in BMD is one of the major factors affecting bone strength, leading to enhanced susceptibility to fractures. BMD is currently one of the reliable indices for the diagnosis of OP, prediction of osteoporotic fractures, and monitoring of its natural course or medication effect.[17] In this study, the results showed that ovariectomy lowered the BMD of the distal femur of female SD rats, while ICA effectively prevented the decline in BMD in ovariectomized rats. ALP is a hydrolase responsible for removing phosphate groups including nucleotides, proteins and alkaloids from many types of molecules, so that phosphate radicals combine with calcium ions to form calcium phosphate minerals deposited in the bone, thereby benefitting bone formation.[18] ALP is a by-product of osteoblast activity, whose content increases during active bone formation.[19] TRAP is a glycosylated monomeric metalloprotease expressed in mammals, which can serve as a marker of bone resorption.

During bone resorption, TRAP can synergize with other enzymes to degrade calcium phosphate minerals in the bone matrix. OC is a kind of mature bone matrix protein synthesized by osteoblasts, involved in the process of bone mineralization. E2 deficiency will result in activation and excessive remodeling of osteoclasts, making postmenopausal women prone to fragility fractures unrelated to bone mass. In this study, OVX group had significantly higher content of serum ALP and TRAP ($P<0.05$), and significantly lower content of E2 and OC than Sham group ($P<0.05$). After ICA treatment, the content of serum ALP and TRAP significantly declined, while the content of E2 and OC rose. The above findings demonstrate that ICA can raise the level of estrogen in the body and regulate the bone metabolic balance.

In addition, in terms of the bone microstructure in each group, ICA can effectively reduce bone mass loss in ovariectomized rats, increase Tb.N, and Tb.Th, reduce Tb.Sp and improve the morphological structure of bone trabecula. Moreover, ICA-H had the most significant effects on the above indices, indicating that ICA-H has the optimal therapeutic effect on OP.

The Notch signaling pathway is one of the key pathways affecting skeletal development, fate, and function of osteoblasts and osteoclasts, which can regulate the differentiation of osteoblasts and osteoclasts, thereby regulating the process of bone remodeling. The Notch receptor family consists of 4 members: Notch1, 2, 3, and 4. Notch ligand is a transmembrane protein with a conserved structure, and there are 5 known Notch ligands (Delta1, 3 and 4, and Jagged1 and 2) in mammals. Jagged1 is one of the main ligands of Notch1, and it binds to Notch1 to lead to continuous proteolytic cleavage mediated by the TADE metalloprotease and γ-secretase complex. Then the Notch signaling pathway is activated, thereby activating the transcription of the downstream target gene Hes family,20 including the main member Hes-1.[21] Cabalis et al. found that the differentiation of osteogenic precursor cells was inhibited when the Notch pathway was activated, thus leading to OP, but when the Notch pathway was suppressed, bone resorption was inhibited but bone formation

was increased.[22]

The results of this study showed that ICA up-regulated the protein expressions of Notch1, Jagged1 and Hes-1, thereby promoting the proliferation and osteogenic differentiation of BMSCs. Hence, ICA can prevent and treat postmenopausal OP through the Notch signaling pathway.

In conclusion, Epimedium extract ICA has a good therapeutic effect on rats with osteoporotic fractures, whose mechanism is related to its ability to up-regulate the protein expressions of Notch1, Jagged1, and Hes-1, inhibit the activity of serum ALP and TRAP, and increase OC, E2 and BMD.

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