Icariin Combined with Adipose-Derived Stem Cells Ameliorated Osteoporosis in Postmenopausal Rats

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Abstract

Purpose: Osteoporosis is a chronic metabolic disease, it has caused the high incidence of related fractures, which seriously affects the life quality of the patients, especially in early postmenopausal women.

Methods: Rat osteoporosis model was induced by bilateral ovarian ablation. Sprague Dawley (SD) rats were randomly divided into five groups (n=9): sham group, model group, the icariin group, ADSCs group, icariin combined with ADSCs group. H&E staining was used to observe the pathological changes of femur. The expression of calcitonin receptor (CALCR) and cathepsin K (CTSK) were investigated by immunohistochemistry. The expression of osteoprotegerin (OPG) and receptor activator of nuclear factor- κ B ligand (RANKL) were measured by qRT-PCR, immunohistochemistry and western blot.

Results: The results showed that icariin combined with ADSCs ameliorated osteoblast morphology and bone density. Icariin combined with ADSCs significantly attenuated the protein expression of CALCR and CTSK after ovariectomy. Moreover, the expressions of OPG mRNA and protein were increased ($p < 0.05$), while the expressions of RANKL mRNA and protein were decreased ($p < 0.05$) in osteoblasts.

Conclusions: Icariin combined with ADSCs can effectively inhibit PMOP in rats. The mechanism may be achieved by up-regulated the ratio of OPG / RANKL.

Introduction

Osteoporosis is a disease which caused by insufficient bone formation. The undifferentiated stem cells fail to differentiate into osteoblasts in time after bone resorption result in insufficient bone formation. In postmenopausal women, the loss of bone matrix and bone mineral content is accelerated in a short period of time, and the degree of systemic bone loss is high, which may easily lead to postmenopausal osteoporosis (PMOP). The effects of various factors such as estrogen levels, cytokines and bone metabolism in the occurrence of PMOP, and the factors are related to each other.¹ The detection of various bone turnover indicators can be important for the prediction of PMOP at the micro level.² Icariin is a small molecule that is the main pharmacological component of *Epimedium*.³ It is a typical flavonol glycoside.⁴ In recent years, study has shown that icariin can significantly reverse bone loss and bone strength in osteoprotegerin (OPG)-deficient mice.⁵ A number of studies have implicated that icariin can increase osteoblast differentiation and inhibit osteoclastogenesis.⁶⁻⁸ In addition, icariin is resistant to dysfunction of bone marrow mesenchymal stem cells (BMSCs) caused by iron overload.⁹

ADSCs can differentiate into chondrocytes, osteoblasts, and glial cells in a specific microenvironment.¹⁰ They are widely used as seed cells and have achieved certain clinical effects.¹¹ Furthermore, ADSCs are widely available, easy to isolated and cultured, and they have the characteristics of immunoregulation and immunosuppression. ADSCs provide favorable conditions for allogeneic transplantation of ADSCs and application in regenerative medicine.^{12, 13}

So far, there are many researches on osteoporosis for *epimedium* and adipose-derived stem cells, respectively,^{9,14,15} the mechanism by icariin combine ADSCs to treat osteoporosis in PMOP rats is unclearly. A previous study have shown that icariin combined with ADSCs can improve bone mass reduction and bone microstructural destruction in osteoporosis rat model, they have protective effects on liver function and kidney.¹⁶ However, the mechanism has not been explored. Therefore, as a further study, we investigated the expression of CALCR and CTSK protein, and the ratio of OPG / RANKL in femur tissue, to explore the mechanism of icariin combined with ADSCs on PMOP rats, and to provide a new theoretical basis for osteoporosis clinical treatment.

Materials And Methods

PMOP Rat Model

Forty-five healthy female SD rats weighted 200 - 220 g were housed in SPF animal room at 20~26 °C and the relative humidity was 50~60%, light and dark cycle was 12/12 h. Rats have free access to eat and drink. The study was approved by the animal care committee, and all experimental operations were conducted according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

After 1 week of adaptive feeding, SD rats were given 1% pentobarbital sodium (40 mg/kg) anesthetized intraperitoneal injection, the skin was disinfected on the back. The longitudinal incision was made in the median, and the skin, subcutaneous, muscular layers were cut open, then a small incision was made into the abdominal cavity on both sides. The bilateral ovaries were completely removed. After surgery, the rats were injected penicillin (Sigma-Aldrich, USA) into the abdominal cavity to prevent infection. All rats were kept in the same condition, fed in a single cage with standard feed. Five days after the operation, the vaginal epithelial cell smear was observed with a cotton swab, and rats with irregular estrus for 5 consecutive days were regarded as successful modeling. ADSCs cells were isolated as described in a previous study.¹⁶

Grouping

A total of 50 rats were randomly selected for bilateral ovarian ablation. Rats were divided into 4 groups randomly (n=9): the model group, the icariin group (250 mg/kg/d, intragastric administration), ADSCs group (ADSCs 2×10^5 cell/ml, intramedullary injection, 0.5ml each time), icariin combined with ADSCs group (icariin + ADSCs group, icariin 250 mg/kg/d, intragastric administration; ADSCs 2×10^5 cell/ml, intramedullary injection, 0.5ml each time). The remaining 9 rats as a sham operation group, all operations were the same as the surgery group, except that the ovaries did not remove. Continuous administration for 12 weeks. After the end of the administration, the rats femur were taken, peeled off attached muscles and connective tissues on the femur, the left side femur was fixed on 10% formaldehyde, and the right side femur was frozen at -20 ° C for use.

Haematoxylin and eosin staining and observation

After the femur was fixed for 24 h in each group, the decalcification was performed by 10% EDTA for 30 days. The decalcification solution was replaced every 3 days, the decalcification of bone tissue was checked during fluid exchange. The femoral tissue was placed in 95% ethanol overnight, gradient dehydration to 100% ethanol, 1 h per gradient, then xylene was transparent, embedded in paraffin, and sectioned 6 μm . Sectional dewaxing with xylene, gradient alcohol rehydration, distilled water soaking, hematoxylin (Solarbio, Beijing, China) staining for 20min, washed in running water back to the blue for 10min, eosin (Solarbio, Beijing, China) staining for 10min, gradient alcohol dehydration, xylene transparent, Neutral gum was used for sealing. Pathological changes in femoral tissue were observed by an optical microscope (magnification, x100; Olympus, Japan).

Immunohistochemistry

The femoral tissue were routine sectioned, the baked flakes were dewaxed with xylene and hydrated sequentially with a gradient ethanol solution. The antigen was repaired in citrate buffer for 20 min, 3% hydrogen peroxide solution was inactivated for 30 min, and blocked with 5% BSA for 20 min. The anti-calcitonin receptor (CALCR) polyclonal antibody (1:20, ab11042, Abcam, UK) and anti-cathepsin K (CTSK) polyclonal antibody (1:1000, ab19027, Abcam), rabbit anti-OPG-antibody (1:50, ab73400, Abcam) and anti-RANKL antibody (1:100, sc52950, Santa Cruz Biotechnology, USA) were added dropwise and reacted at 4 °C overnight. After rewarming, the sections were incubated with the horseradish peroxidase-labeled goat anti-rabbit IgG and anti-mouse IgG (Abcam, UK) at 37 °C for 1 h. DAB (Solarbio, Beijing, China) was used for color development, hematoxylin was lightly counterstained, dehydrated, transparent, and sealed. Three sections of each tissue were observed under a 100 $\times$ optical microscope (Olympus, Japan) to analyze positive cell.

QRT-PCR

The right femur of each group of rats was extracted RNA by TRIzol Reagent (Invitrogen, USA) after grinding. RNA purity and integrity were detected by UV spectrophotometer (Thermo Fisher Scientific, USA) at A260 /280. Reverse transcription was performed by TaKaRa Primescript TM RT ReagentKit (TaKaRa, Japan). 10 μL cDNA configuration reaction system was used to perform the PCR reaction by the SYBR Premix Ex Taq (TaKaRa, Japan). The primer sequences were as follows: OPG: (Forward) 5'-TGGACAACCCAGGAAACCTTTCCTCCAAAA-3' (Reverse) 5'-TTTGCCTGGGACCAAAGTGAATGCAGAGAG-3'; RANKL: (Forward) 5'-GCCAGCCGAGACTACGGCAAGTACCTGCGC-3', (Reverse) 5'-GGCCAGGTGGTCTGCAGCATCGCTCTGTTC-3'; β -actin: (Forward) 5'-GTGG GGATAATGAACTTGCAAG-3', (Reverse) 5'-GGAACCCCTGGTAGAACAGT-3'. The PCR reaction was performed under the following conditions: 95°C for 30 sec, 95°C for 5 sec, 60°C for 30sec, 30 cycles, and incubation at 72 °C for 10 min. Data were processed using the $2^{-\Delta\Delta\text{Ct}}$ method¹⁷ and relative expression levels were calculated using β -actin mRNA as an internal reference.

Western blot assay

The right femur of each group of rats was extracted and the tissue protein was extracted after grinding. The concentrations of RANKL and OPG protein were sequentially determined according to the method of BCA Protein Quantitation Kit (Thermo Fisher Scientific, USA). Each group of sample was loaded with 40 µg, separated by SDS-PAGE and transferred to a PVDF membrane (Millipore, USA). The membrane was blocked with 5% skim milk for 1 h, then primary antibody anti-OPG antibody (1:1000, sc390518, Santa Cruz Biotechnology, USA), anti-RANKL antibody (1:1000, sc52950, Santa Cruz Biotechnology, USA) were added and incubated at 4 °C overnight. TBST (TBS, 1 ml/L Tween-20) was used to wash the membrane for 3 times, 5 min/time. HRP-labeled secondary goat anti-mouse IgG (1:5000, sc2005, Santa Cruz Biotechnology, USA) was added and incubated for 2 h at room temperature. The membrane was washed with TBST three times, 10 min/time; ECL chemiluminescence was developed in darkroom. The protein expression levels were normalized to β-actin and quantified by Image J 1.46 (National Institutes of Health, USA) software.

Statistical analysis

IBM SPSS statistical software 19.0 was used to perform the statistical analysis. All experimental data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to comparison between groups, subsequent analysis was performed by LSD test. Statistical significant difference was assumed at $p < 0.05$.

Results

Pathological examination of femur tissue in each group

Microscopic observation showed that the bone tissue of the rats in the sham operation group was normal, the bone tissue was regular, the cytoplasm was rich and had good morphology, the intercellular tube was intact, and the bone cells were distributed in the trabecular bone and cortical bone. The cells were flat and cuboidal, covered the surface of the bone and arranged in a string (Fig. 1A). In the model group, the arrangement of bone cells were disordered, the bone cells number and density were significantly decrease compared with the sham group, and the intercellular tubule connection mostly disappeared. The osteoblasts were cuboidal or elliptical, showed the empty sag after osteoblast apoptosis (Fig. 1B). The morphology of the icariin group and the ADSCs group were similar, the bone cells number and density were significantly increased (Fig. 1C, 1D). The bone cells in the icariin + ADSCs group were normal. The cytoplasm was abundant, and the morphology was basically well. The osteoblasts were arranged in a cuboidal shape on the surface of the bone.

The CALCR expression in femoral tissue

The result of IHC showed that the positive expression of CALCR in the sham group was the weakest (Fig. 2A), number of CALCR positive cells in model group was significantly increased (Fig. 2B, 2F). The positive expression of CALCR in the icariin group was similar to the ADSCs group, number of positive cells compared to model group were decreased (Fig. 2C, 2D). In the icariin+ ADSCs group, number of CALCR

positive cells were significantly attenuated compared to model group, and there was no significant difference with the sham group (Fig. 2E).

The CTSK expression in femur tissue

As shown in Fig.3, the positive expression of CTSK in the sham was the weakest (Fig. 3A), number of CTSK positive cells in model group was significantly increased (Fig. 3B). The positive expression of CTSK in the icariin group was similar to the ADSCs group, number of positive cells compared to model group were decreased (Fig. 3C, 3D, 3F). In the icariin+ ADSCs group, number of CTSK positive cells were obviously attenuated than that in model group, and there was no difference from the sham group (Fig. 3E).

Expression of RANKL and OPG mRNA and protein in femur of each group

The relative number of IHC positive cells in each group was analyzed (Figure 4). OPG in model group was significantly lower than that in sham group, while the RANKL was higher in model group. The relative OPG positive cell number in icariin group and the ADSCs group were significantly higher than that in model group, and it was the highest in icariin+ ADSCs group. In contrast, RANKL was significantly decreased in icariin group and the ADSCs group, it was the lowest in icariin+ ADSCs group.

The results of qRT-PCR, and western blot showed that the expression of RANKL mRNA and protein in the model group femur tissues were significantly increased compared with the sham group, while the OPG mRNA and protein were significantly decreased ($p < 0.05$) (Figure 5). After the treatment, the expression of RANKL in the icariin group and the ADSCs group were significantly decreased, respectively, compared with the model group. In contrast, the OPG mRNA and protein expression was significantly increased ($p < 0.05$). The effect of the icariin+ ADSCs group was the most significant compared with the treatment with icariin or ADSCs alone.

Discussion

Epimedium, a traditional Chinese medicine, has attracted a lot of research on its active ingredients by the effect of “reinforcing the kidney, strengthening the muscles and strengthening the bones”. It is now clear that the main active ingredient of anti-osteoporosis is represented by icariin. The flavonoid compound,¹⁸ its mechanism of action is to promote osteogenic differentiation of bone marrow mesenchymal stem cells, and has dual activities to promote bone formation and inhibit bone resorption.¹⁹ Therefore, we thought that icariin can promote ADSCs differentiate into osteoblasts in a specific microenvironment.

Calcitonin is an important hormone that regulates calcium metabolism and bone turnover in the body.²⁰ CALCR is mainly distributed in osteoclasts and their precursor cells in bones, it can regulate bone metabolism and maintain calcium balance in the body.²¹ Calcitonin specifically binds to CALCR on osteoclasts, inhibits the activation of monocytes to osteoclasts, and the number of osteoclasts, accordingly slowing bone resorption and delaying bone loss. CTSK is involved in the pathogenesis of

various diseases and has the closest relationship with osteoporosis. It is the most important cytokine found in osteoclasts, with the highest expression level and the strongest osteolytic activity. It is involved in the degradation of extracellular matrix.²² In bone degradation, activated osteoclasts secrete acidic substances to dissolve minerals on the bone surface, while collagen or bone matrix is also degraded by CTSK secreted by osteoclasts.²³ Studies have shown that the excessive collagenase activity of CTSK in osteoclasts is the main cause of excessive degradation of bone matrix and osteochondral collagen, which leads to osteoporosis.²⁴ In our study, the results of immunohistochemistry showed that icariin combined with ADSCs can significantly inhibited the expression of CALCR and CTSK in femoral tissue, inhibiting bone deterioration caused by ovariectomy.

OPG/RANK/RANKL is one of the most important molecular systems involved in regulating bone remodeling.²⁵ OPG is a type of transmembrane protein synthesized by osteoblasts. With the differentiation of osteoblasts, OPG can inhibit bone resorption, increase cortical bone, cancellous bone density, area and bone strength.²⁶ RANKL binds to the osteoclast surface receptor RANK, promotes osteoclast formation, differentiation, maturation, inhibits osteoclast apoptosis, and prolongs its survival.^{27,28} OPG prevents RANKL and RANK interaction by binding to RANKL in adjacent osteoclasts, thereby inhibiting osteoclast formation, differentiation, survival, activation, and induction of osteoclast apoptosis.²⁹ Study has shown that the analysis of OPG/RANKL ratio can reflect the osteogenic effect, osteoclast effect and bone turnover state. The larger the ratio, the more obvious the osteogenic effect.³⁰ Our results indicated that icariin combined with ADSCs increases OPG mRNA and protein expression in osteoblasts and decreases RANKL mRNA and protein expression in osteoclasts. It was further explained that the mechanism of action of icariin combined with ADSCs to inhibit osteoporosis in postmenopausal rats may be achieved by up-regulating OPG/RANKL and promoting osteogenic effects.

Some reports investigated that mainly activated T lymphocytes and antigen presenting cells, can also express RANKL, therefore influencing bone remodelling, both directly and through cytokine production.³¹⁻³³ Autophagy plays an important role in cell proliferation, differentiation, apoptosis and maintenance of cell homeostasis, especially in the process of promoting bone formation and promoting bone resorption.^{34,35} It is critical to PMOP. The autophagy of cells in the human body is universal, and the changes in the level of autophagy in PMOP may not only involve estrogen levels and oxidative stress, but also gradually increase with the body's age. In the beginning of aging in the body can generally induce autophagy, but the inhibition of autophagy can also promote aging, which may be the promotion of cell aging by autophagy in the resistance to the accumulation of harmful substances reaching a certain limit and severely damaged.³⁶ In this study, we only explored the effect of icariin combined with ADSCs on OPG/RANKL. In the future, we need to further explore the direction of immune cells and autophagy.

In conclusion, further studies have shown that icariin combined with ADSCs can inhibited the expression of CALCR and CTSK in femoral tissue, up-regulated the OPG/RANKL ratio, promoted osteogenesis, and thus reduced bone deterioration. Provide theoretical basis for the treatment of postmenopausal osteoporosis patients.

Conclusions

Through the above experiments, combined with previous studies, we concluded that icariin combined with ADSCs showed significant therapeutic effects in PMOP rats. The possible mechanism may be icariin combined with ADSCs can inhibit the expression of CALCR and CTSK, up-regulate OPG/RANKL ratio in femoral tissue, promote osteogenic effect, decrease the osteoclast differentiation thereby reducing bone deterioration and achieving therapeutic goals.

Declarations

Acknowledgments

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Availability of data and materials

Data collected or analyzed during this study are included in this article.

Ethics approval and consent to participate

This study was approved by Ethics committee of the Yantai Hospital of Traditional Chinese Medicine.

Consent for publication

Not applicable.

Conflicts of interest statement

Ying Sun, Dongbo Wang, Dao Tang, Yanjie Liu, Fei Xu, Zhengguang Wang and Ziqi Liu have stated that they have no conflicts of interest.

Contributions

ZY, WD and LZ designed the study; WD, TD, LY, XF and WZ performed experiments; ZY, WD, TD, LY and XF performed data analysis; TD, LZ, WD, WZ, LY and ZY contributed pathological analysis; TD and LZ interpreted the data; WD, TD, LY, XF, WZ and LZ aided with sample acquisition; ZY, WD and LZ wrote the manuscript; All authors approving the final version of the manuscript.

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Figures

Figure 1

Pathological changes of the femur tissue. (magnification $\times 200$, Scale bar: 100 μm) A: sham group; B: model group; C: icariin group; D: ADSCs group; E: icariin + ADSCs group.

Figure 2

Immunohistochemical detection of CALCR protein expression in femur tissue. (magnification $\times 200$, Scale bar: 100 μm) A: sham group; B: model group; C: icariin group; D: ADSCs group; E: icariin + ADSCs group; F: CALCR coverage. ** $p < 0.01$ compared with the sham group; * $p < 0.05$ compared with the sham group; # $p < 0.05$ compared with the model group; $\square p < 0.05$ compared with the icariin or ADSCs group.

Figure 3

Immunohistochemical detection of CTSK protein expression in femur tissue. A: sham group; B: model group; C: icariin group; D: ADSCs group; E: icariin + ADSCs group; F: CTSK coverage. ** $p < 0.01$ compared with the sham group; * $p < 0.05$ compared with the sham group; # $p < 0.05$ compared with the model group; $\square p < 0.05$ compared with the icariin or ADSCs group.

Figure 4

Immunohistochemical detection of OPG and RANKL expression. A: Immunohistochemical detection of OPG protein; B: Percentage of OPG positive cells; C: Immunohistochemical detection of RANKL protein; D: Percentage of RANKL positive cells. ** $p < 0.01$ compared with the sham group; * $p < 0.05$ compared with the sham group; # $p < 0.05$ compared with the model group; □ $p < 0.05$ compared with the icariin or ADSCs group.

Figure 5

The expression of OPG and RANKL mRNA and protein. A: The quantification of OPG mRNA expression; B: The quantification of RANKL mRNA expression. C: Western Blot assay detected the protein expression; D: quantification of OPG protein; E: quantification of RANKL protein.* $p < 0.05$ compared with the sham group; # $p < 0.05$ compared with the model group; $\square p < 0.05$ compared with the icariin or ADSCs group.