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Ipriflavone promotes osteogenesis of MSCs derived from osteoporotic rats

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Abstract. – OBJECTIVE: To explore whether Ipriflavone could prevent postmenopausal osteoporosis (PMOP) and improve bone quality via promoting osteogenesis of bone marrow-derived mesenchymal stem cell (MSCs).

MATERIALS AND METHODS: MSCs were extracted from rats and identified using flow cytometry. Osteogenic specific genes and adipogenic specific genes in MSCs were detected by quantitative Real-time polymerase chain reaction (qRT-PCR). The effect of Ipriflavone on osteogenesis was detected by CCK-8 (cell counting kit-8) assay, ALP activity detection, alizarin red staining and Western blot, respectively. Furthermore, ovariectomized PMOP rat model was constructed. The effects of Ipriflavone on osteogenesis, BMD and bone biomechanical properties of ovariectomized rats were detected.

RESULTS: MSCs derived from ovariectomized rats exerted multiple differentiation potentials. CCK-8 assay indicated that 0.8 μM Ipriflavone were the maximal dose that did not affect MSCs proliferation, which was selected for the following experiments. In vitro researches demonstrated that Ipriflavone remarkably promoted MSCs osteogenesis. *In vivo* results indicated that BMD, BV/TV, Tb.N and Tb.Th were decreased in ovariectomized rats than those of rats in sham group. Ipriflavone treatment remarkably prevented osteoporosis via promoting MSCs osteogenesis in ovariectomized rats.

CONCLUSIONS: Ipriflavone prevents postmenopausal osteoporosis, improves bone quality and protects bone tissue via promoting MSCs osteogenesis.

Key Words:
Ipriflavone, MSCs, Osteogenesis, Osteoporosis.

Introduction

Postmenopausal osteoporosis (PMOP) is a common disease in postmenopausal women[1], which is manifested as ovarian dysfunction[2] and reduced estrogen secretion[3,4]. PMOP leads to accelerated bone resorption, bone mass decrease, microstructure destruction and bone fragility elevation, thus eventually leading to high risk of fracture[5]. Therefore, PMOP is a serious problem that poses a great challenge in public health[6]. In recent years, hormone replacement therapy and calcitonin intervention are the main treatments for PMOP[7]. However, the long-term use of estrogen significantly increases the prevalence of endometrial cancer and breast cancer[8]. Besides, the long-term use of calcitonin injection is inconvenient and expensive[3]. Currently, Ipriflavone has been well recognized since it could effectively prevent PMOP[9,10].

Ipriflavone is a phytoestrogenic drug derived from isoflavones. However, Ipriflavone has no estrogenic effect in the human body[11]. It has been shown that Ipriflavone can directly act on the bone and inhibits bone resorption[12]. Meanwhile, Ipriflavone stimulates calcitonin release from the thyroid gland, thereby exerting therapeutic effects as estrogen and calcitonin but without their side effects[13]. Therefore, Ipriflavone has a promising prospect in preventing PMOP.

In this study, we constructed ovariectomized rat model of PMOP to observe the effect of Ipriflavone on MSCs differentiation, BMD, and bone biomechanical properties. Our results provide solid basis for the better application of Ipriflavone in clinical prevention and treatment of PMOP.

Materials and Methods

Isolation and Culture of MSCs

3-month-old female Sprague Dawley (SD) rats weighing from 280-300 g were obtained from the Model Animal Research Center of Nanjing University. After one-week adaptive feeding, rats were randomly assigned into sham group, ovariectomy group (OVX group)

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and ovariectomy + Ipriflavone treatment group (OVX + Ipriflavone group), with 20 rats in each group. 8 weeks later, rats were sacrificed with dislocation of cervical vertebra. The femur and tibia were collected under aseptic condition, washed with 75% ethanol for 10-30 s and D-Hanks solution for 5-8 times. The marrow cavity was washed with α-MEM (α-Modified Eagle Medium) containing 10% fetal bovine serum (FBS). After centrifugation at 1000 r/min for 5 min, MSCs were re-suspended inα-MEM and maintained in a 5% CO_2 incubator at 37°C (Gibco, Rockville, MD, USA). MSCs were then seeded in 6-well plates at a density of 1.0×10^9/L. Half of culture medium was replaced 24 h later and total medium was replaced after 48 h. Third-passage MSCs were collected for the following studies. All animal experiments were approved by The First Affiliated Hospital of Soochow University Ethics Committee.

Ovariectomized PMOP Rat Model

Rats were anesthetized with intraperitoneal injection of 1% pentobarbital sodium (4 mL/kg) and placed on the surgical table. A midline incision was cut to expose the abdominal cavity. Bilateral ovariectomy was performed in rats of OVX group and OVX + Ipriflavone group. The abdominal cavity was sutured layer by layer. Rats in sham group were only cut with a small piece of adipose tissue near the ovary. All rats recovered well after the procedures.

MSCs Identification

MSCs identification was performed using flow cytometry. In brief, MSCs were resuspended in PBS (phosphate-buffered saline) at a density of 1.0×10^6/mL. After washing with PBS twice, 500 μL of suspension were incubated with 5 μL of CD45 and CD73 at room temperature without light for 30 min. After PBS washing for three times, cells were centrifuged and resuspended in 500 μL of PBS, followed by flow cytometric detection.

Osteogenic Differentiation of MSCs

Third-passage MSCs were seeded in the 6-well plates at a density of 2.0×10^4 per well. After the cell confluence was up to 70-80%, osteogenic differentiation was induced by α-MEM containing 10 mmol/L sodium β-glycerophosphate, 50 μg/mL Vitamin C, 10 μmol/L dexamethasone and 100 mL/L FBS. Culture medium was replaced every 3 days for a total of 14-21 d.

Lipid Differentiation of MSCs

Third-passage MSCs were seeded in the 6-well plates at a density of 2.0×10^4 per well. Lipid differentiation was induced by α-MEM supplemented with 0.5 mmol/L 3-isobutyl-1-methylxanthine (IBMX), 5 mg/L insulin and 1 μmol/L dexamethasone for 3 days. On the 4th day, MSCs were cultured with Dulbecco's Modified Eagle Medium (DMEM) containing 5 mg/L insulin for a total of 14-21 d.

RNA Extraction and qRT-PCR (Quantitative Real-Time Polymerase Chain Reaction)

The TRIzol kit (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA, followed by reverse transcription into complementary Deoxyribose Nucleic Acid (cDNA). QRT-PCR was performed to detect the expressions of related genes according to the instructions of 2 × SYBR Green PCR MasterMix (Thermo Fisher Scientific, Waltham, MA, USA). PCR reaction was performed as follows: denaturalization at 95°C for 15 s, extension at 60°C for 15 s and annealing at 72°C for 32 s, for a total of 40 cycles. Each experiment was repeated in triplicate. The relative gene expression was calculated as $2^{\Delta\Delta Ct}$. Primers used in the study were: ALP, F: 5'-TTCCACAACAGGCCACTTACT-3', R: 5'-GAGCATCTCTGCTTGTCTATCTG-3'; Bglap, F: 5'-CACTCCTCGCCCTATTGGC-3',R:5'-CCCTCCTGCTTGGACACAAAG-3'; RUNX2, F: 5'-TGGTTACTGTCATGGCGGGTA-3', R: 5'-TCTCAGATCGTTGAACCTTGCTA-3'; PPARG, F: 5'-GGGATCAGCTCCGTGGATCT-3', R: 5'-TGCACTTTGGTACTCTTGAAGTT-3'; CEBPA, F:5'- CCGCCTCAGTGATTTAGGGC-3', R: 5'-GGGTCTGTAATCTGACTCTGTCC-3';KLF5, F: 5'-TCAGTCGTAGACCAGTTCTTCA-3', R: 5'-CTGGGATTTGTAGAGGCCAGT-3'; GAPDH, F: 5'- ACCCACTCCTCCACCTTTGA-3', R: 5'-CTGTTGCTGTAGCCAAATTCGT-3'.

CCK-8 (Cell Counting Kit-8) Assay

MSCs treated with different doses of Ipriflavone were seeded in the 96-well plates at a density of 1.0×10^4 per well. After culturing for 24 h, 10 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) were added into each well. The absorbance values at the wavelength of 450 nm were detected by the microplate reader (Bio-Rad, Hercules, CA, USA).

ALP Activity Detection

MSCs were seeded in the 6-well plates at a dose of 1.0×10^8/L. Each group had 6 replicates.

After cell culture with serum-free α-MEM for 24 h, complete α-MEM supplemented with different doses of Ipriflavone was added into each well. 72 h later, cells were washed with D-Hanks and incubated with 1 mL of lysate at 4°C for 30 min. ALP activity was then detected according to the instructions of the commercial kit.

Western Blot

The total protein of MSCs was extracted by the radioimmunoprecipitation assay (RIPA) lysate (Yeasen, Shanghai, China). The concentration of each protein sample was determined by a BCA (bicinchoninic acid) kit (Abcam, Cambridge, MA, USA). Briefly, total protein was separated by a 12% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) gel under denaturing conditions and transferred to PVDF (polyvinylidene difluoride) membranes (Merck Millipore, Billerica, MA, USA). Membranes were blocked with 5% skimmed milk for 1 h, followed by the incubation of specific primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight. After washing with TBST (Yeasen, Shanghai, China) for 3 times, membranes were then incubated with the secondary antibody (Cell Signaling Technology, Danvers, MA, USA) at room temperature for 1 h. Immunoreactive bands were exposed by enhanced chemiluminescence method.

Alizarin Red Staining

Third-passage MSCs were seeded in the 6-well plate pre-coated with slices at a dose of 1.0×10^5 / mL. MSCs were treated with different doses of Ipriflavone, with 3 replicates in each group. After Ipriflavone treatment for 21 days, cells were washed with PBS, fixed with 95% ethanol for 30 min and stained 0.1% alizarin red for 30 min. The amount of calcified nodules was observed and captured using an inverted microscope (40×).

Detection of BMD and Trabecular Structure Indicators

The left femur of rat was weighed and maintained in the 4% neutral formalin. Femur samples were preserved in PBS 48 h before detection. Microarchitecture was detected using micro-CT (50 kV of scanning voltage, 50 μA of current, 35 μm of resolution power and 360° rotation), and analyzed using Skyscan software (CT Analyser, Skyscan, Brussels, Belgium). Relative indicators in ROI (region of interest) were detected, including BMD (bone mineral density), BV/TV (bone volume/total volume), Tb.N (trabecular number) and Tb.Th (trabecular thickness).

Statistical Analysis

We used Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) for statistical analysis. The quantitative data were represented as mean ± standard deviation ($\bar{x}\pm s$). The *t*-test was used for comparing differences between the two groups. $p<0.05$ was considered statistically significant.

Results

Identification of MSCs Purity and Multiple Differentiation Potential

Elongated MSCs were observed using a microscope. The typical morphology of MSCs on the 2nd and 3rd day were shown in Figure 1A. Third-passage MSCs were collected for surface antigen identification. The data showed negative-antigen CD45 (3.2%) and positive-antigen CD73 (99.97%), indicating the high purity of extracted MSCs (Figure 1B). Subsequently, mRNA levels of osteogenic specific genes and adipogenic specific genes were detected by qRT-PCR. The data elucidated that ALP, Bglap, and RUNX2 were remarkably upregulated in a time-dependent manner after osteogenesis for 3, 7 and 14 days (Figure 1C). The mRNA levels of PPARG, CEBPA and KLF5 were also increased in a time-dependent manner after lipid differentiation for 1, 7 and 14 days (Figure 1D). The above results demonstrated the extracted MSCs exert a high purity and multiple differentiations potential.

Ipriflavone Promoted MSCs Osteogenesis

We first detected the effects of different doses of Ipriflavone on MSCs proliferation by CCK-8 assay. The results showed that 0.8 μM is the maximal dose without affecting cell proliferation. Therefore, 0.4 μM and 0.8 μM Ipriflavone were selected for the following experiments (Figure 2A). MSCs were induced with osteogenic medium containing different doses of Ipriflavone for 7 days. The mRNA levels of ALP, Bglap and RUNX2 were elevated in a dose-dependent manner (Figure 2B). Besides, ALP activity in MSCs treated with 0.4 μM and 0.8 μM Ipriflavone was increased in a dose-dependent manner (Figure 2C). Protein expression of RUNX2 showed the similar increased trend (Figure 2D). Alizarin red staining results suggested a significant calcifica-

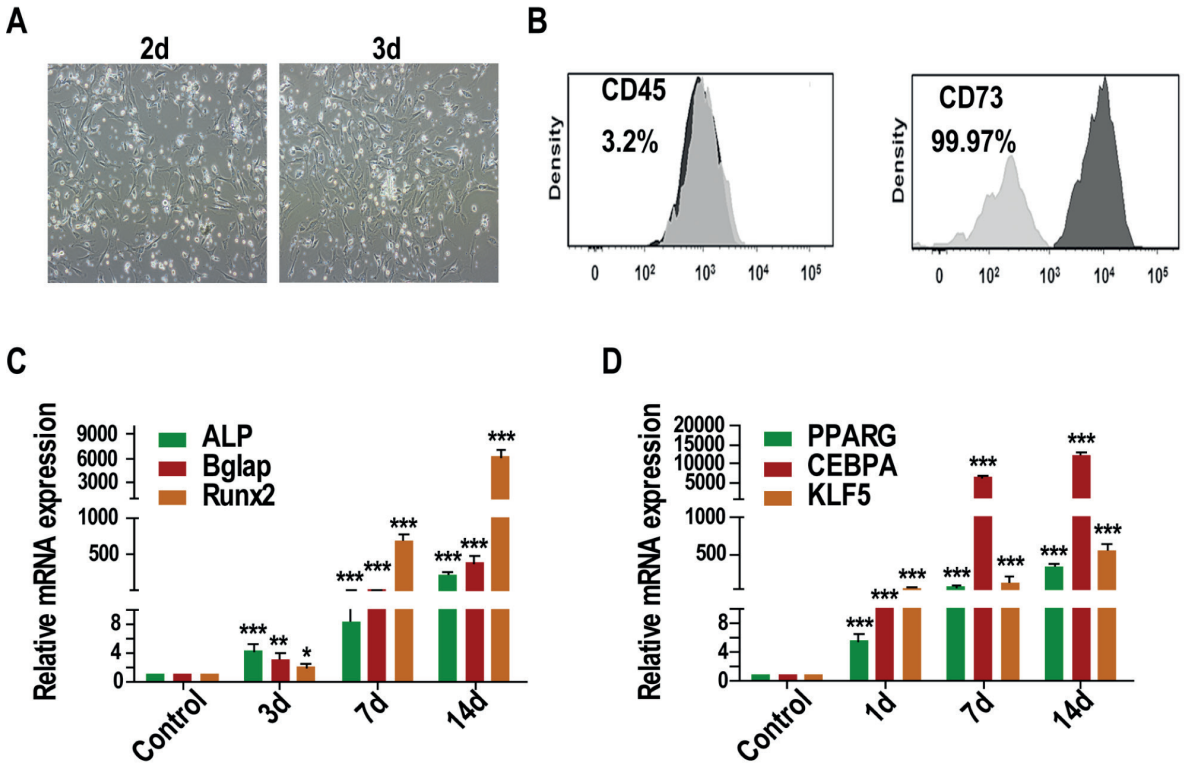

Figure 1. Identification of MSCs purity and multiple differentiation potential. ***A,*** The typical morphology of MSCs on the 2nd and 3rd day. ***B,*** Flow cytometry data showed negative-antigen CD45 (3.2%) and positive-antigen CD73 (99.97%) in third-passage MSCs. ***C,*** ALP, Bglap and RUNX2 were remarkably upregulated in a time-dependent manner after osteogenesis for 3, 7 and 14 days, respectively. ***D,*** The mRNA levels of PPARG, CEBPA and KLF5 were increased in a time-dependent manner after lipid differentiation for 1, 7 and 14 days.

tion in MSCs after Ipriflavone treatment (Figure 2E). Our results indicated that Ipriflavone remarkably promotes MSCs osteogenesis.

Ipriflavone Increased Bone Mass of Ovariectomized Rats

To further explore the biological effect of Ipriflavone, we constructed ovariectomized PMOP rat model. The data elucidated that BMD (Figure 3A), BV/TV (Figure 3B), Tb.N (Figure 3C), and Tb.Th (Figure 3D) in rats of OVX group were lower than those of sham group. However, these indicators were all higher in OVX + Ipriflavone group compared with those of OVX group, indicating that Ipriflavone remarkably increased bone mass of ovariectomized rats.

Ipriflavone Promoted MSCs Osteogenesis in Ovariectomized Rats

MSCs were extracted from rats of sham group, OVX group and OVX+ Ipriflavone group, respectively. After osteogenic induction for 7 days, expression levels of ALP, Bglap and RUNX2 in MSCs extracted from rats of OVX group were lower than those of sham group and OVX+ Ipriflavone group (Figure 4A). ALP activity and protein expression of RUNX2 were also detected in the three groups. The decreased ALP activity and RUNX2 expression induced by ovariectomy were partially reversed by Ipriflavone treatment (Figure 4B and 4C). Alizarin red staining results suggested that Ipriflavone promotes mineralizing ability of MSCs extracted from ovariectomized rats (Figure 4D).

Discussion

With the aging of population, the health of postmenopausal women has caused the concern of the whole society. Decreased serum level of estrogen in postmenopausal women results in bone loss. Hence, estrogen exerts a very important role in the maintenance of bone mass[14]. Currently, es-

trogen replacement therapy is considered to be the preferred treatment for PMOP, which remarkably elevates BMD in postmenopausal women[15]. Although estrogen can effectively treat and prevent PMOP, accumulating studies[16] have confirmed that long-term use of estrogen increases risks of breast cancer, stroke, myocardial infarction, cholecystitis, and thrombosis. Phytoestrogens have similar structures to that of 17β-estradiol, which are natural selective estrogen receptor modulators. Therefore, phytoestrogen is of clinical significance in preventing and reducing bone loss as an estrogen replacement during peri-menopausal period[17-20].

Ipriflavone is a type of synthetic isoflavone phytoestrogens that can effectively increase bone

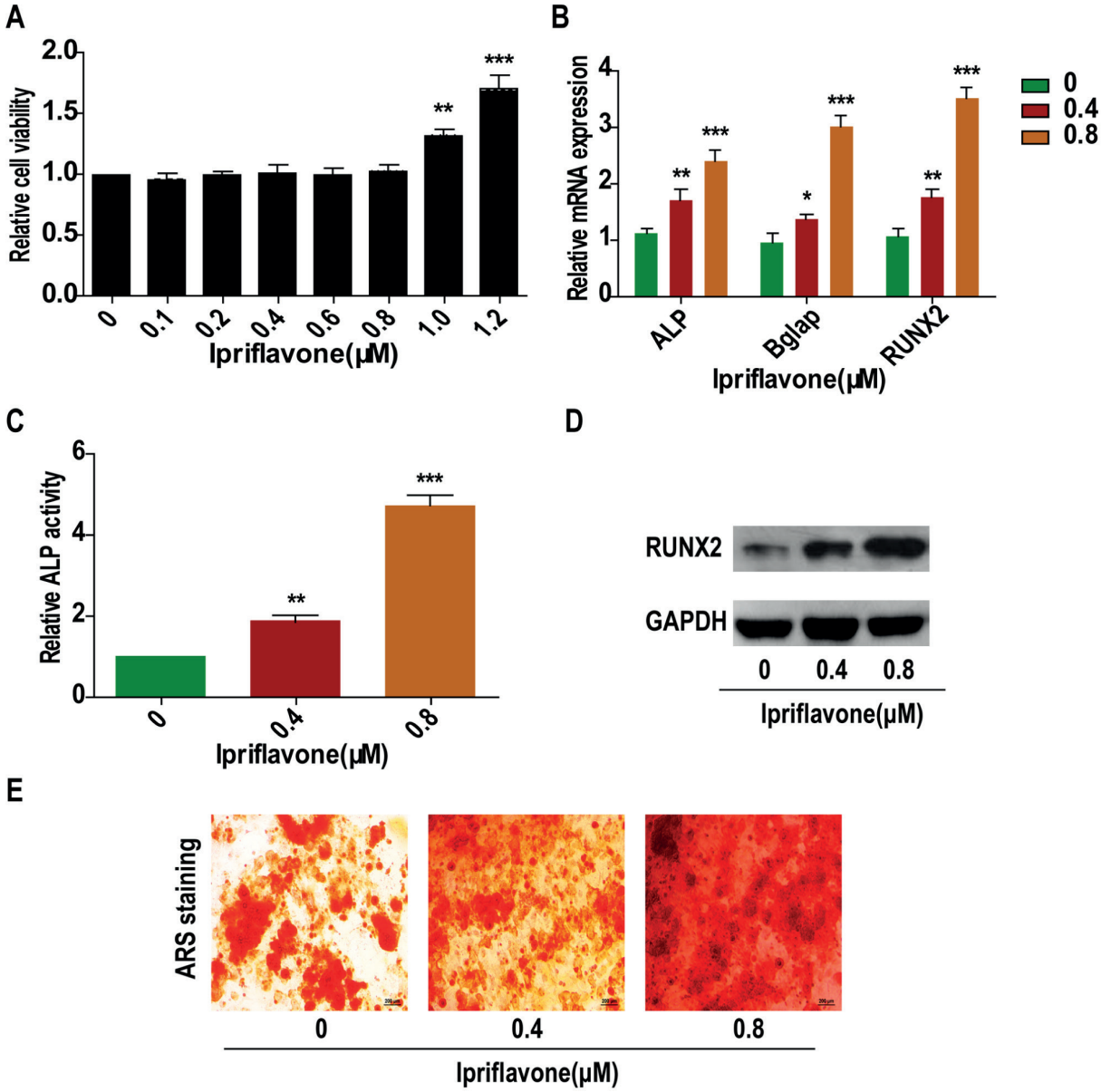

Figure 2. Ipriflavone promoted MSCs osteogenesis. ***A,*** CCK-8 results showed that 0.8 μM is the maximal dose without affecting cell proliferation. ***B,*** The mRNA levels of ALP, Bglap and RUNX2 were elevated in a dose-dependent manner after MSCs were induced with osteogenic medium containing different doses of Ipriflavone for 7 days. ***C,*** ALP activity in MSCs treated with 0.4 μM and 0.8 μM Ipriflavone was increased in a dose-dependent manner. ***D,*** Protein expression of RUNX2 in MSCs treated with 0.4 μM and 0.8 μM Ipriflavone was increased in a dose-dependent manner. ***E,*** Alizarin red staining results suggested an obvious calcification in MSCs after Ipriflavone treatment.

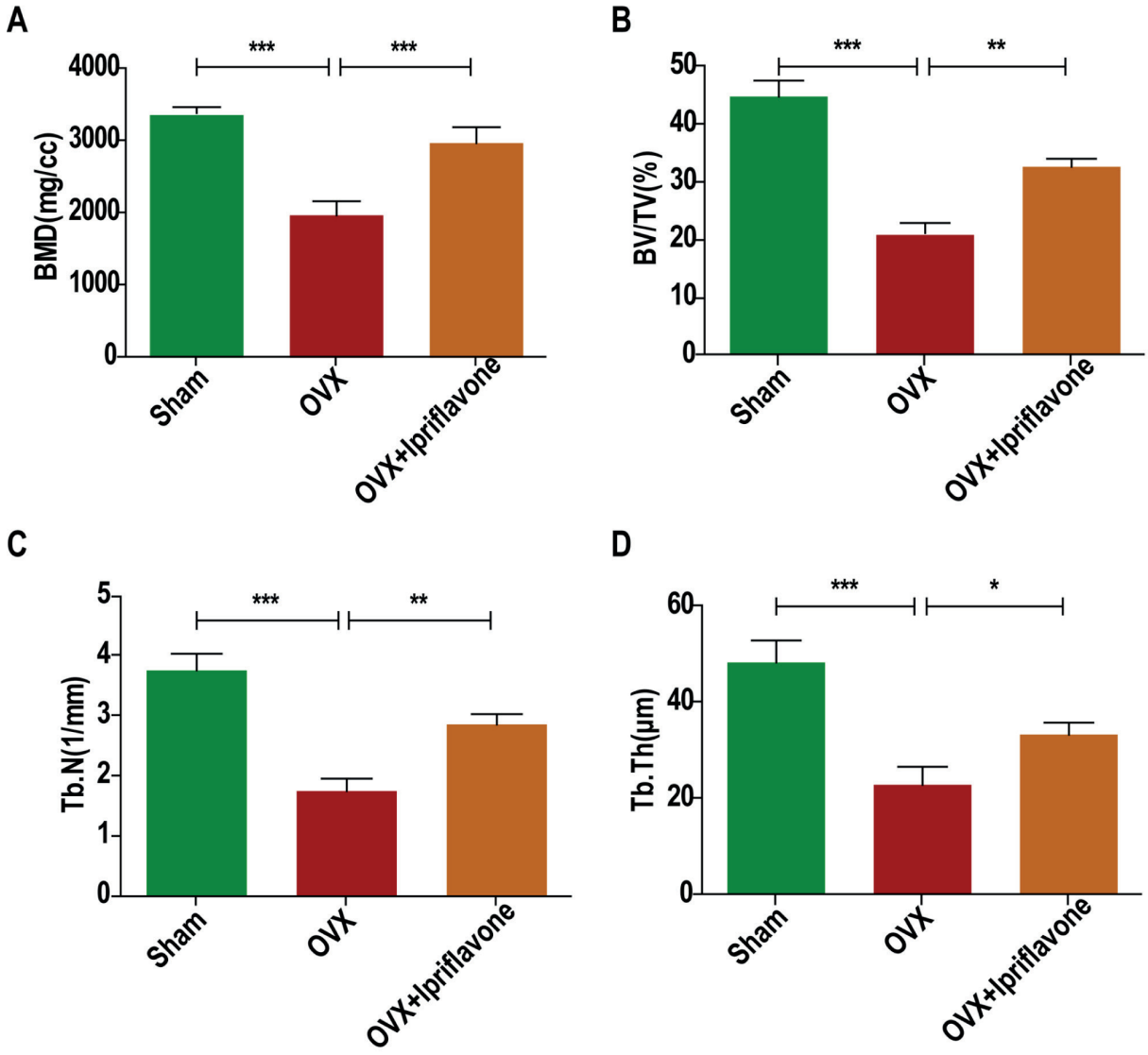

Figure 3. Ipriflavone increased bone mass of ovariectomized rats. ***A-D,*** BMD (***A***), BV/TV (***B***), Tb.N (***C***) and Tb.Th (***D***) in rats of sham group, OVX group and OVX + Ipriflavone group.

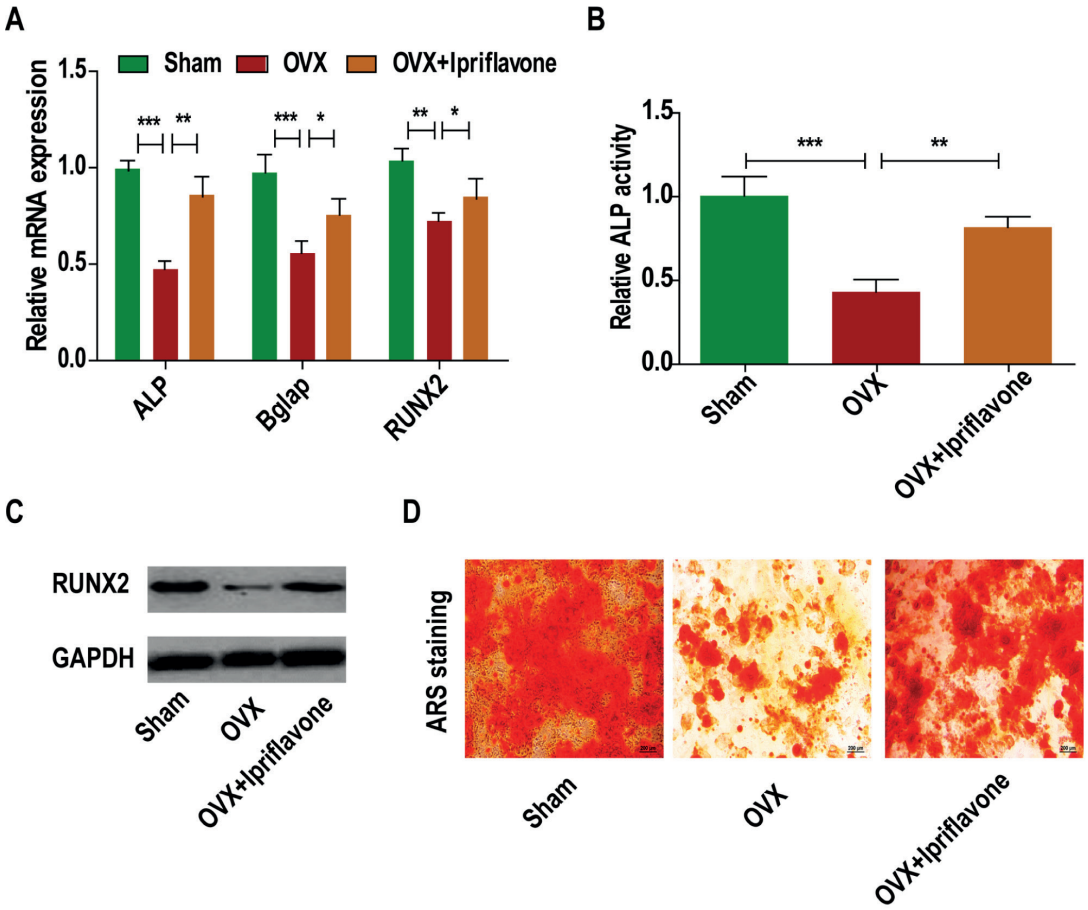

Figure 4. Ipriflavone promoted MSCs osteogenesis in ovariectomized rats. MSCs were extracted from rats of sham group, OVX group and OVX+ Ipriflavone group, respectively and underwent osteogenic induction for 7 days. ***A,*** The mRNA levels of ALP, Bglap and RUNX2 in the three groups. ***B,*** ALP activity in the three groups. ***C,*** Protein expression of RUNX2 in the three groups. ***D,*** Alizarin red staining results in the three groups.

density in postmenopausal women[21,22]. Arjmand et al[22] found that Ipriflavone can efficiently prevent ovariectomy-related bone loss. The protective effect of Ipriflavone on estrogen-deficient bone loss is different from that of estrogen effect. It mainly protects bone loss via increasing bone formation rate. Deyhim et al[23] found that Ipriflavone could not increase bone density and inhibit bone resorption. It protects bone quality via inhibiting bone loss.

In the present study, we first extracted rat MSCs and induced osteogenic differentiation with Ipriflavone. Subsequently, BMD, BV/TV, Tb.N and Tb.Th were found to be decreased in ovariectomized rats than those of rats in sham group. Ipriflavone treatment remarkably prevented osteoporosis via promoting MSCs osteogenesis in ovariectomized rats.

Conclusions

We showed that ipriflavone prevents postmenopausal osteoporosis, improves bone quality and protects bone tissue via promoting MSCs osteogenesis.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References

1) Kamel HK. Postmenopausal osteoporosis: etiology, current diagnostic strategies, and nonprescription interventions. J Manag Care Pharm 2006; 12: S4-S9.

2) Korkmaz V, Kurdoglu Z, Alisik M, Turgut E, Sezgin OO, Korkmaz H, Ergun Y, Erel O. Thiol/disulfide

homeostasis in postmenopausal osteoporosis. J Endocrinol Invest 2017; 40: 431-435.

3) Tella SH, Gallagher JC. Prevention and treatment of postmenopausal osteoporosis. J Steroid Biochem Mol Biol 2014; 142: 155-170.

4) Chen YY, Wang WW, Yang L, Chen WW, Zhang HX. Association between lipid profiles and osteoporosis in postmenopausal women: a meta-analysis. Eur Rev Med Pharmacol Sci 2018; 22: 1-9.

5) Li X, Jie Q, Zhang H, Zhao Y, Lin Y, Du J, Shi J, Wang L, Guo K, Li Y, Wang C, Gao B, Huang Q, Liu J, Yang L, Luo Z. Disturbed MEK/ERK signaling increases osteoclast activity via the Hedgehog-Gli pathway in postmenopausal osteoporosis. Prog Biophys Mol Biol 2016; 122: 101-111.

6) Chen X, Zhi X, Cao L, Weng W, Pan P, Hu H, Liu C, Zhao Q, Zhou Q, Cui J, Su J. Matrine derivate MASM uncovers a novel function for ribosomal protein S5 in osteoclastogenesis and postmenopausal osteoporosis. Cell Death Dis 2017; 8: e3037.

7) Fan JZ, Yang L, Meng GL, Lin YS, Wei BY, Fan J, Hu HM, Liu YW, Chen S, Zhang JK, He QZ, Luo ZJ, Liu J. Estrogen improves the proliferation and differentiation of hBMSCs derived from postmenopausal osteoporosis through notch signaling pathway. Mol Cell Biochem 2014; 392: 85-93.

8) Kharroubi A, Saba E, Smoom R, Bader K, Darwish H. Serum 25-hydroxyvitamin D and bone turnover markers in Palestinian postmenopausal osteoporosis and normal women. Arch Osteoporos 2017; 12: 13.

9) Ito K, Minegishi T, Takayama T, Tamura T, Yamada Y, Sato S. Effects of ipriflavone on augmented bone using a guided bone regeneration procedure. Clin Oral Implants Res 2007; 18: 60-68.

10) Delmas PD. Treatment of postmenopausal osteoporosis. Lancet 2002; 359: 2018-2026.

11) Halpner AD, Kellermann G, Ahlgrimm MJ, Arndt CL, Shaikh NA, Hargrave JJ, Tallas PG. The effect of an ipriflavone-containing supplement on urinary N-linked telopeptide levels in postmenopausal women. J Womens Health Gend Based Med 2000; 9: 995-998.

12) Belcavello L, Vencioneck DJ, de Freitas JV, Aranha IP, Do CPBM. Mutagenicity of ipriflavone in vivo and in vitro. Food Chem Toxicol 2012; 50: 996-1000.

13) Lv WT, Yang YH, Ma LQ, Wang P, Li K. Ipriflavone reverses the adverse effects of a low-calcium diet on the histology of the tibia in caged layers. Br Poult Sci 2014; 55: 207-214.

14) Reginster JY, Neuprez A, Beaudart C, Lecart MP, Sarlet N, Bernard D, Disteche S, Bruyere O. Antiresorptive drugs beyond bisphosphonates and selective oestrogen receptor modulators for the management of postmenopausal osteoporosis. Drugs Aging 2014; 31: 413-424.

15) McClung MR, Boonen S, Torring O, Roux C, Rizzoli R, Bone HG, Benhamou CL, Lems WF, Minisola S, Halse J, Hoeck HC, Eastell R, Wang A, Siddhanti S, Cummings SR. Effect of denosumab treatment on the risk of fractures in subgroups of women with postmenopausal osteoporosis. J Bone Miner Res 2012; 27: 211-218.

16) Vitolo E, Comassi M, Caputo MT, Solini A. Hormone replacement therapy, renal function and heart ultrasonographic parameters in postmenopausal women: an observational study. Int J Clin Pract 2015; 69: 632-637.

17) Alwerdt J, Valdes EG, Chanti-Ketterl M, Small BJ, Edwards JD. The relationship between phytoestrogens and speed of processing. Menopause 2016; 23: 911-918.

18) von Mach-Szczypinski J, Stanosz S, Kosciuszkiewicz J, Safranow K. New aspects of postmenopausal osteoporosis treatment with micronized estradiol and progesterone. Ginekol Pol 2016; 87: 739-744.

19) Lagari VS, Levis S. Phytoestrogens for menopausal bone loss and climacteric symptoms. J Steroid Biochem Mol Biol 2014; 139: 294-301.

20) Rietjens I, Louisse J, Beekmann K. The potential health effects of dietary phytoestrogens. Br J Pharmacol 2017; 174: 1263-1280.

21) Alexandersen P, Toussaint A, Christiansen C, Devogelaer JP, Roux C, Fechtenbaum J, Gennari C, Reginster JY. Ipriflavone in the treatment of postmenopausal osteoporosis: a randomized controlled trial. JAMA 2001; 285: 1482-1488.

22) Arjmandi BH, Birnbaum RS, Juma S, Barengolts E, Kukreja SC. The synthetic phytoestrogen, ipriflavone, and estrogen prevent bone loss by different mechanisms. Calcif Tissue Int 2000; 66: 61-65.

23) Deyhim F, Smith BJ, Soung DY, Juma S, Devareddy L, Arjmandi BH. Ipriflavone modulates IGF-I but is unable to restore bone in rats. Phytother Res 2005; 19: 116-120.