Mongolian Medicine echinops prevented postmenopausal osteoporosis and induced ER/AKT/ERK pathway in BMSCs

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Summary
Hormone replacement medicine such as traditional Chinese medicine has proven to be effective in decreasing the risk of osteoporosis. Mongolian medicine echinops prevents osteoporosis, but its mechanism remains unclear. In this study, we explored the mechanism underlying echinops prevents and treats postmenopausal osteoporosis. Osteoporosis model was established by ovariectomy in rats. Rats were treated to Echinops (16.26, 32.5, or 65 mg/kg/day) by oral gavage for 3 months. Bone mineral density (BMD) was detected by micro-CT detection of left proximal medial metaphyseal tibia. Hematoxylin and eosin (H&E) and toluidine blue O staining were also performed. Serum levels of E2, ALP and testosterone were examined. Bone marrow-derived bone marrow stem cells (BMSCs) were isolated and treated with echinops-containing serum. Estrogen receptors (ER) including ERα and ERβ in bone specimens and BMSCs were detected by qRT-PCR. Cell viability and colony formation of BMSCs were detected. Results showed that echinops significantly increased trabecular interconnectivity, thickness of trabeculae, and connection of trabecula. Echinops significantly increased BMD and E2, but significantly reduced ALP and testosterone in dose-dependent manners. Echinops induced ERα and ERβ in both bone specimens and BMSCs. Echinops enhanced cell viability and ability of colony formation of BMSCs, and increased ERα, ERβ, p-AKT, and p-ERK. Thus, Mongolian echinops reduced bone loss and delayed the occurrence and development of osteoporosis, and increased ERα, ERβ, p-AKT, and P-ERK in BMSCs. These results provide experimental basis for clinical prevention and treatment of postmenopausal osteoporosis by echinops.

Keywords: Osteoporosis, echinops, ERα, ERβ, AKT/ERK pathway

1. Introduction
Osteoporosis (OP) is a metabolic bone disease characterized by low bone mass and the destruction of the microstructure of bone tissue, leading to increased bone fragility and easy to fracture (1,2). OP is the sixth most common chronic diseases in humans (3). It was divided into two types: primary OP and secondary OP (4,5). Primary OP can be divided into two subtypes, namely Type I and Type II. Type I is postmenopausal osteoporosis (PMOP), which occurs in postmenopausal women (6,7). Type II is senile osteoporosis, most commonly seen in the elderly over 60 years old. About 200 million people worldwide suffer from osteoporosis, of which postmenopausal women account for 1/3 (8,9). The overall incidence of OP in Chinese population over 60 is 22.6%, with 15% for males and 28.6% for females, with a trend of increasing year by year (10). The incidence of OP in the United States is also quite high, about 20,000 cases of OP fractures, and 65,000 cases

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died due to OP each year (11).

PMOP occurs in 5 to 10 years after menopause in women, most of whom have an increased bone turnover rate, due to fluctuations or gradually reduced in the level of estrogen. In post-menopausal 5-7 years, women lose about 20% of the bone mass (12). Although most osteoporosis does not directly cause death, its greatest risk is fractures, with high morbidity and disability (6,11). Estrogen deficiency caused by postmenopausal ovarian hypofunction is recognized as an important cause of postmenopausal osteoporosis (13-15). Estrogen replacement therapy is the preferred method of treatment for PMOP, which can enhance bone mineral density (BMD) and systemic bone mineral content, and effectively treat postmenopausal osteoporosis (16-18). However, the long-term use of estrogen increases the risk of breast cancer, endometrial cancer, cardiovascular accident and vascular embolism (19). In recent years, the prevention and treatment of PMOP by traditional Chinese medicine is attracting more and more attention (20,21). It is of great significance to find an estrogen replacement medicine for the prevention and treatment of postmenopausal osteoporosis in traditional medicine.

Mongolian medicine echinops was introduced in the canon of Mongolian Medical "Wisdom Ancientmirror". It functions as strengthening bone, reuniting bone, and callus (22). In recent year, it was showed that echinops decreased the serum level of bone Glp protein and inhibited osteoporosis in ovariecetomized (OVX) rats (23). Post-surgery 90 days, the OVX rats were filled the stomach with echinops for 90 days and then the serum level of alkaline phosphatase (ALP) were significantly increased and serum level of interleukin-1 was significantly reduced (24). After successes of the osteoporosis, OVX rats were feed 90 days and then filled the stomach with echinops for 90 days, then BMD and the maximum deflection of bone were increased compared with OVX rats (25). These results implied that Mongolian echinops can inhibit the bone absorption and promote the bone formation, decreasing bone turnover, reducing bone loss, delaying the occurrence and development of PMOP. However, the mechanism underlying echinops prevents and treats PMOP is still unclear.

In this study, we aimed to explore the roles of estrogen receptors (ER; ERα and ERβ), p-AKT, and p-ERK in BMSCs during echinops prevents and treats postmenopausal osteoporosis.

2. Materials and Methods

2.1. Animals and treatments

A total of 84 SPF healthy female Wistar rats (250 ± 20 g, 4 months) were purchased from the animal research center of Inner Mongolia University, China (certification number: SCXK (Mongolia) 2012-0001). The rats received ad libitum access to standard chow pellets and water in 24°C, 50-60% humidity. This study was approved by animal ethics committee of Inner Mongolia University. After 7 days in new environment, the rats were anesthetized by intraperitoneal injection of 40 mg/kg pentobarbital sodium (P3761, SIGMA-ALDRICH, USA), shaved off the hair on the bilateral dorsal regions for OVX surgery (26). The ovaries were exposed by a 2 mm incision and resected with surgical scissors. Then, other exposed tissues were repositioned and incision was sutured with 3.0 silk threads in a routine fashion. Intrapertitoneal injection of penicillin was administrated. Rats in sham groups was incision without ligation of ovaries artery.

Post-surgery 3 months (26), animals were treated to echinops (16.26, 32.5, or 65 mg/kg/day) by oral gavage for 3 months as previously reported (25). Echinops was prepared as below: weigh 500 g of dried echinops at 75°C, mixed with 10-time water, decoted for 1 h and filtrated; the slag was decocted with same volume water and filtrated again; the two filtration solutions were collected and concentrated to 500 ml to obtain 1g/mL echinops stock solution. The rats in OVX and sham groups received PBS daily. E2 treatment (E2758, SIGMA-ALDRICH) was set as positive control. After 3 months, the rats were euthanized by intraperitoneal injection of 40 mg/kg pentobarbital sodium, whole blood was collected from the heart through cardiac puncture, and the femur medial malleolus specimens were selected at 1 mm under the epiphyseal plate. After centrifuged at 3,000 rpm for 10 min, serum samples were collected, filtered with 0.22 μm filter, and stored at -20°C for subsequent experiments.

2.2. Hematoxylin and eosin (H&E) and toluidine blue O staining

The femur bones were fixed in 10% neutral buffered formalin solution for 48 h, dehydrated in graded ethanol (70-100%, cleared in xylene, embedded in paraffin, and sectioned into 5 μm. For H&E staining, sections were stained with hematoxylin for 3-8 min and eosin for 1-3 min. For toluidine blue O staining, sections were rinsed in toluidine blue O solution for 1 min. The images were observed by Olympus BX51 light microscopy (Olympus, Japan).

2.3. Micro-computed tomography (micro-CT) detection

Micro-CT of left proximal medial metaphyseal tibia were acquired using Scanco Mct35 scanner (Scanco, Switzerland) at 70 KVP, 114 μA for 800 ms. Bone mineral density was evaluated based on the micro-CT results.

2.4. Serum levels of E2, ALP, and testosterone

After centrifuged at 3,000 rpm for 10 min, serum samples
were collected and stored at -20°C until enzyme-linked immunosorbent assay (ELISA) detection. The levels of E2 (CSB-E05108h, CUSABIO), ALP (A059-2, Nanjing Jiancheng Bioengineering Institute), and testosterone (05099h, CUSABIO) were determined by commercially ELISA kits according to manufacturer's instruction using an Multiskan microplate reader (Thermo, USA).

2.5. qRT-PCR detection

Total RNA from bone specimens were extracted using Trizol (Takara, Japan). The RNA quality and quantity were examined using Nanodrop 1000 spectrophotometer (NanoDrop, USA). RT reaction was performed with Bestar qPCR RT kit (ABI, USA) according to manufacturer’s instruction on ABI9700 PCR system (ABI, USA). The PCR reaction was performed using DBI Bestar® SybrGreen qPCRmasterMix (ABI, USA) on Stratagene Mx3000P Real time PCR system (Agilent, USA) according to manufacturer’s instruction. The primer was listed below (5’-3’): R-GADPH Forward CCTCGTCTCTAGACAAAGATGT, reversed GGTTAGAGTCTAATCGGAACATG; ERa Forward AAGAAGAATAGCCCCCCTTGT, reversed GCCAGGTTGGAATTCCGCCAG; ERβ Forward ATGCGCTGTCTGGGTGAT, reversed CCCCGAGATTGAGGACTTGT. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control. Relative expression was calculated using 2-ΔΔCt method and normalized to sham group.

2.6. Bone marrow-derived bone marrow stem cell (BMSC) isolation and flow cytometry identification

The rat femur was rinsed in PBS containing 1% penicillin-streptomycin. After removal of both ends in joints, the bone was rinsed in DMEM with low glucose by a syringe until the bone pale. The mediums were collected and centrifuged at 800 rpm for 5 min. Cells were resuspended into DMEM with low glucose and cultured in incubator at 37°C, 5%CO2. After 48 h, cells were stained with primary antibodies of CD29 (ab179471, Abcam, USA), CD90 (ab216449, Abcam), CD11b (ab128797, Abcam), and FITC-conjugated secondary antibody (ab6717, Abcam) in the darker for 30 min. The CD29, CD90, CD11b positive cells were analyzed using Epics-XL II flow cytometry (Beckman Coulter, USA).

2.7. Cell viability and colon formation

Cell viability was detected with a cell counting-8 kit (CCK-8, Beyotime, China). Cells (5x105 cells) were seeded in 96-well plates and incubated with echinops-containing serum for 24, 48 and 72h. Then, CCK-8 was added and the absorbance was detected at 450 nm on microplate reader. For colon formation assay, cells were seeded in 6-well plates at 8 × 104 cells/well with echinops-containing serum for 13 days. Then, cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet.

2.8. Western blotting

Protein was extracted using RIPA (Beyotime, China) with PMSF (1:100) at 4°C for 30 min, and quantified by BCA assay (#23227, Thermo, USA). 30 μg protein was separated by 8%SDS-PAGE and transferred to PVDF membranes (IPVH00010, Millipore, USA). The membrane was incubated with 5% non-fat milk for 30 min, and incubated with primary antibodies ERα (1:2,000), ERβ (1:4,000), AKT (1:1,500), p-AKT (1:1,000), ERK (1:1,000), p-ERK (1:2,000), and GAPDH (1:10,000) antibody at room temperature for 1 h, and then incubated with HRP goat anti-rabbit IgG secondary antibody (1:20,000, BOSTER, China) at room temperature for 40 min. The blots were detected using Immobilon Western CHEMILUM HRP Substrate (WBKLS0500, Millipore, USA), and light-producing reactions are captured with X-ray film.

2.9. Statistical analysis

Data were expressed as mean ± standard deviation, and compared using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. Statistical analyses were performed using the SPSS 10.0 software (SPSS, USA). Significance was considered at p < 0.05.

3. Results

3.1. Echinops increased BMD and E2, but reduced ALP and testosterone in dose-dependent manners

BMD at 90 days post-treatment was analyzed by micro-CT (Figure 1A). Compared with sham group, a significant reduction in BMD of cortical bone in O VX group was observed (p < 0.01). 16.25 mg/kg echinops not significantly increased the trabecular BMD, whereas 32.5 mg/kg (p < 0.05), 65 mg/kg (p < 0.001) and E2 significantly increased the BMD of trabecular bone in comparison to the untreated OVX rats.

The rats in OVX group showed lower level of E2 (p < 0.01, Figure 1B), and higher levels of ALP (p < 0.01, Figure 1C) and testosterone (p < 0.01, Figure 1D) than sham group. 16.26 mg/kg echinops not significantly changed the levels of E2 and testosterone, but significantly inhibited ALP level (p < 0.01) in comparison to the OVX group. 32.5 mg/kg and 65 mg/kg echinops significantly increased E2 level but decreased levels of ALP and testosterone in OVX rats (Figure 1B-D). There was not significant difference in ALP level between 32.5 mg/kg and 65 mg/kg treatment groups (Figure 1C).

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Thus, echinops increased BMD and level of E2, but decreased levels of ALP and testosterone in concentration-dependent manners.

3.2. Echinops induced expression of ERα and ERβ

To detect the role of echinops on ERα and ERβ expression, qRT-PCR assays were performed (Figure 2). Compared with sham group, a significant decrease in ERα and ERβ mRNA expressions were shown in OVX rats (Figure 2A and B). Echinops administrated at 16.26, 32.5 and 65 mg/kg significantly increased ERα and ERβ expressions in comparison to OVX group, showing a dose-dependent manner (Figure 2A and B).

3.3. Echinops inhibited osteopenia induced by OVX

After surgery for 90 days (26), rats in sham and OVX group were treated with echinops (32.5 mg/kg) or E2 for 90 days. Then, rats were euthanized and bones were collected. HE and toluidine blue O stainings showed a typical osteopenia with widened intertrabecular spaces, loss of trabecular bone thickness and interconnectivity in OVX group, compared with sham group (Figure 3). Echinops or E2 treatment significantly increased trabecular interconnectivity, thickness of trabeculae, and connection of trabecula, compared with OVX group, suggesting echinops inhibited the osteopenia induced by OVX (Figure 3).

3.4. Isolation and identification of BMSCs

To explore the mechanism in echinops treated osteoporosis, BMSCs were isolated and identified by flow cytometry of CD29, CD90, CD45, and CD11b. There were 90.6% isolated-cells positively expressed CD29 (Figure 4A), 93.8% positively expressed CD90 (Figure 4B), but only 4.5% positively expressed CD45 (Figure 4C), only 1.3% positively expressed CD11b (Figure 4D). These results suggested the isolated cells were almost BMSCs.

3.5. Echinops enhanced cell viability and ability of colony formation of BMSCs

To evaluate role of echinops in cell proliferation of BMSCs, cell viability (Figure 5A) and colony formation (Figure 5B and C) were performed. Echinops-containing serum (65 mg/kg) significantly increased cell viability (Figure 5A). Moreover, echinops-containing serum significantly increased colony formation of BMSCs in a dose-dependent manner ($p < 0.01$ at 16.26 and 32.5 mg/kg, and $p < 0.001$ at 65 mg/mg, Figure 5B). These
results suggested echinops-containing serum induced cell proliferation of BMSCs in a dose-dependent manner.

3.6. Echinops increased ERα, ERβ, p-AKT, and P-ERK in BMSCs

To explore the mechanism in echinops treated osteoporosis, expressions of ERα, ERβ, AKT, p-AKT, ERK, and p-ERK in BMSCs after echinops-containing serum treatment were examined (Figure 6). After treatment of Echniops-containing serum, ERα and ERβ levels were significantly increased compared with control BMSCs (Figure 6A and B). Moreover, the phosphorylations of AKT and ERK were significantly induced in a dose-dependent manner (Figure 6C and D).

4. Discussion

In this study, it was demonstrated that echinops functions like estrogen. It can effectively prevent and treat PMOP. Administrating echinops to ovariectomy-induced PMOP model, the BMD and serum level of E2 were increased, serum levels of ALP and testosterone were decreased. Echinops induced expression of ERα and ERβ in OVX rats. The mechanism in echinops prevented PMOP was explored by treating isolated BMSCs with echinops-containing serum. Echinops-containing serum significantly increased cell viability and colony formation of BMSCs, and increased ERα, ERβ, p-AKT, and P-ERK in BMSCs.

Rats are the most used model animal in the studies of osteoporosis so far (14,27). After ovariectomy in female rats, bone turnover accelerated, bone loss and bone strength decreased, which was similar to that of people after menopause (28). Using OVX model, it is

figure 4. Identification of BMSCs isolated from rats. Flow cytometry detection of (A) CD29, (B) CD90, (C) CD45 and (D) CD11b on isolated cells were performed.

figure 5. Effects of echinops-containing serum on cell viability and colony formation. BMSCs were treated with Echniops-containing serum that collected from rats administrated with 16.26 (low), 32.5 (middle), and 65 (high) mg/kg echinops. (A) Cell viability. (B, C) Colony formation. *p < 0.05, **p < 0.01, ***p < 0.001 vs control.

figure 6. Effects of echinops-containing serum on expressions of ERα, ERβ, AKT, p-AKT, ERK, and p-ERK in BMSCs. BMSCs were treated with Echniops-containing serum that collected from rats administrated with 16.26 (low), 32.5 (middle), and 65 (high) mg/kg echinops. (A) ERα mRNA. (B) ERβ mRNA. (C) ERα, ERβ, AKT, p-AKT, ERK, and p-ERK. (D) Quantification of western bolts. *p < 0.05, **p < 0.01 vs control.
easy to observe the effect of aging on bone tissue, the
distribution and reconstruction of cancellous bone in
rats and trabecular bone reconstruction of lamellar bone
that similar to human (28). In the present study, a PMOP
animal model was established in ovariectomized rats.
The ovaries were artificially removed and the estrogen
deficiency was induced in rats. The ERα and ERβ levels
were also decreased. After 3 months, osteoporosis model
was successfully replicated in OVX rats as previously
described (26).

Estrogen is recognized drug in the prevention and
treatment of PMOP, and diethylstilbestrol (E2) is a
synthetic non-steroidal estrogen which can produce
pharmacological and therapeutic effect similar to natural
estradiol, and significantly reduce the ovariectomy-
induced high bone turnover and -reduced bone resorption
(29,30). Therefore, this study selected E2 as a positive
control drug to validate the mechanism of Mongolian
medicine echinops in PMOP treatment. Similar effects to
E2 was demonstrated in this study.

After menopause, women's estrogen levels decreased
significantly, estrogen through the estrogen receptor (ER)
directly effect on the osteoblast and osteoclasts, lead
to imbalance of bone resorption and bone formation,
resulting in reduced bone mass and BMD, increased
bone fragility and the occurrence of osteoporosis (31,32).
The decrease of estrogen level and the decrease of the expression
of ER in bone tissue are one of the most
important pathogenesis. Estrogen can directly stimulate
osteoblasts formation, inhibit osteoclasts activity, and
regulate and control the balance of bone formation and
bone resorption through ER. ER is expressed in both
osteoblasts and osteoclasts (33,34). Osteoclasts is a very
active metabolic giant multinucleated cells, recruited
in the bone surface, and played important role in bone
resorption and formation of lacuna through release of
enzyme and acidic substances such as ALP to dissolve
the bone matrix (34,35). Osteoblasts can synthesize
the basic bone material, induces the formation of bone
(33). The combination of estrogen and ER in osteoclasts
induces the apoptosis of osteoclasts and osteoclasts
precursors, reduces the number of osteoclasts. On the
other hand, the combination of estrogen and ER in
osteoclasts inhibited the recruitment and differentiation
of osteoclasts precursors. In addition, estrogen regulated
by osteoprotegerin/osteoprotegerin ligand (OPG/OPGL)
system (36). The decline in estrogen levels results in a
dysregulated ratio of osteoprotegerin/osteoprotegerin
ligands leading to PMOP (37).

It was demonstrated that echinops decreased the serum
level of bone Glp protein, interleukin-1, but
increased serum level of ALP, increasing BMD, and
inhibiting osteoporosis in ovariectomized (OVX) rats
(23-25). Activation of ERK/PI3K plays important role in
ER-mediated cell proliferation (38). Daidzein stimulated
osteogenesis was mediated by both ERα and ERβ,
and activation of ERK/PI3K pathway (39). Consistently, the
findings in this study showed echinops reduced ALP and
testosterone serum levels in OVX rats, increased BMD
and inhibited osteoporosis. In isolated BMSCs, echinops
induced cell proliferation, increased ERα, ERβ, p-AKT,
and P-ERK. This might associate with the enhancement of
osteoblasts differentiation from BMSCs, inhibiting
bone absorption and promote bone formation.

In conclusion, Mongolian echinops reduced bone loss
and delayed the occurrence and development of PMOP,
and increased ERα, ERβ, p-AKT, and p-ERK in BMSCs.
These results provide experimental basis for clinical
prevention and treatment of PMOP by echinops.

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