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Ameliorative effects of *Schizandra chinensis* on osteoporosis via activation of estrogen receptor (ER)- α / β

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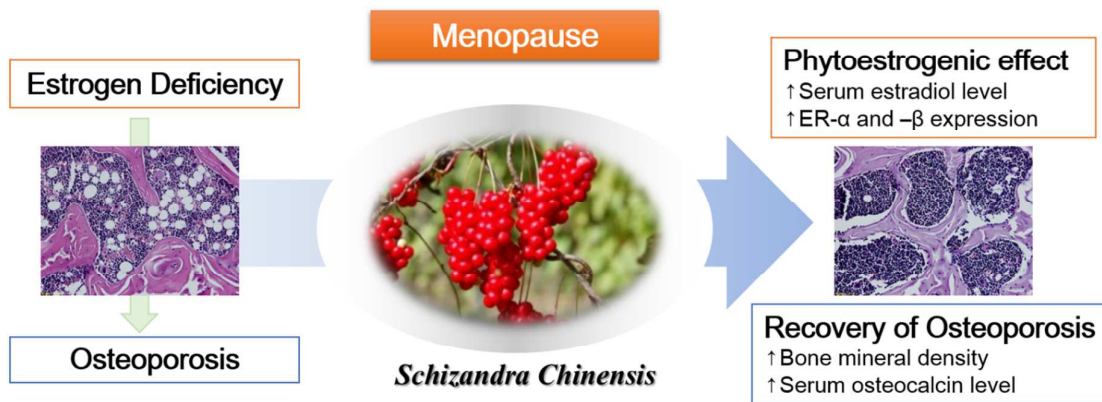
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Graphical abstract



Abstract

Estrogen deficiency in menopausal women is the main cause of osteoporosis. Phytoestrogen could be a treatment for post-menopausal osteoporosis. Recent studies showed that *S. chinensis* contains several lignans, which may be phytoestrogen. In this study, we investigated the ameliorative effects of *S. chinensis* on post-menopausal osteoporosis.

30% ethanol extract of *S. chinensis* (SC) was administrated orally for 6 weeks along after 7 weeks of ovariectomized-induced osteoporosis. Bone mineral density was significantly increased following increased serum osteocalcin levels by SC treatment. Histological analysis showed that SC reduced the increased growth plate of the epiphyseal plate in femur. In addition, pore within bone marrow cells filling the lateral and medial epicondyle were decreased. Serum estradiol concentration was significantly increased in SC-treated group. The expressions of estrogen receptor- α and - β were increased in uterus and MCF-7 breast cancer cells by SC treatment. And two transcription of proto-oncogenes, c-fos and c-Jun, were suppressed by treatment of SC.

From these data, we propose that *S. chinensis* attenuates post-menopausal osteoporosis with its phytoestrogenic effects. *S. chinensis* may have the potential to be used as an alternative treatment for osteoporosis.

Keywords: *Schizandra chinensis*; Osteoporosis; Menopause; Phytoestrogen

Introduction

Menopause occurs as part of the biological aging process in females. In menopause, estrogen production in the ovaries is interrupted and maintained at a low level due to aromatase inhibitors^{1,2}. Estrogen deficiency leads to a variety of menopausal symptoms; hot flashes, sweating, anxiety, depression, mood swings, sleep disorders, vaginal dryness and joint pain³. Rapid bone loss is also caused by estrogen deficiency, resulting in the occurrence of post-menopausal osteoporosis.

Increase in bone remodeling and imbalance between bone resorption and formation after menopause leads to a decrease in bone mass and bone mineral density (BMD)¹. Two types of anti-osteoporotic drugs, those preventing bone resorption (estrogen, calcitonin, bisphosphonates, raloxifene), and those stimulating bone formation (fluoride, anabolic steroids), are used for bone remodeling⁴. Hormone replacement therapy (HRT) has been used to prevent and treat osteoporosis for decades⁵. However, due to the long-term effects of HRT and the risk of breast cancer, medicinal herb-based phytoestrogen is a possible alternative treatment of osteoporosis⁶.

Phytoestrogens are estrogen-like compounds of plant origin that act as selective estrogen receptor modulators when binding to estrogen receptors (ER) instead of estrogen⁷. Phytoestrogen-rich herbs are capable of treating osteoporosis^{6,8}. The fruit of *Schizandra chinensis* (Turcz.) Baill. is used as functional foods and traditional medicinal herbs for rheumatism and arthritis⁹. *S. chinensis* includes some lignan compounds, such as shizandrin, deoxyschizandrin, γ -schizandrin, gomisins A and gomisins N, which were reported to stimulate the proliferation and activity of bone cell differentiation marker in osteoblast cells^{7, 10-12}. Schizandrin inhibited human breast cell proliferation via regulation of the cell cycle and

related proteins¹³. There is potential that *S. chinensis* may be a possible anti-osteoporosis agent with minimal cancer risk. However, it has not been investigated the efficacy of *S. chinensis* on osteoporosis yet.

In this study, a 30% ethanol extract of *S. chinensis* was administrated to ovariectomized (OVX) mice and incubated in MCF-7 cells. To determine the phytoestrogenic effect of *S. chinensis* on osteoporosis, we evaluated BMD, bone histopathology, serum estradiol and osteocalcin *in vivo*, and ER expression *in vitro*.

Materials and Methods

Preparation of SC

Schizandra chinensis BAALL. (Family: Magnoliaceae) was obtained from Mungyeong-si, Gyeongnam, Republic of Korea. The fruit of *S. chinensis* was extracted in 30% ethanol for 2 h at 100°C by refluxing. The extract was filtered, concentrated in a rotary vacuum evaporator, and lyophilized (called SC). The obtained powder (yield: 27%) was kept at -20°C until use. A voucher specimen (SC-E30) was deposited at our laboratory.

The concentration of schizandrin in SC was determined by high-performance liquid chromatography diode array detector (HPLC-DAD, Agilent 1260 Infinity). Chromatographic separation was achieved using the C18 column (Zorbax Eclipse Plus C18 4.6 × 250 mm, 5 µm). The eluent for schizandrin, gomisins A and gomisins N consisted of acetonitrile, water and formic acid (70:30:0.1 v/v) at a flow rate of 0.8 ml/min. The detection wavelength was 254

nm.

Ovariectomy-induced animals and treatment

Female ICR mice (6 weeks old; 20 ± 2 g) were purchased from Central Lab Animal Inc. (Seoul, Republic of Korea). Mice were acclimatized for 1 week. All mice were provided free access to a standard chow diet (Orient, Seongnam, Republic of Korea) and tap water. They were maintained at 22 ± 2 °C, with a relative humidity of $50 \pm 5\%$ and a 12 h light-dark cycle. The animal studies were conducted in accordance with the rules and regulations established by the Institutional Animal Ethics Committee of Kyung Hee University.

The mice were randomly divided into six groups ($n = 6$); Normal, OVX + vehicle, OVX + E2, OVX + 1 mg/kg SC, OVX + 10 mg/kg SC, and OVX + SC 100 mg/kg. After adaption, mice except normal group were surgically ovariectomized. The mice were allowed to recover and induce osteoporosis for 7 weeks. The normal and OVX + vehicle group mice were orally administered the vehicle (PBS containing 1% DMSO). The OVX + E2 (as a positive control) were injected intraperitoneally E2 (10 μ g/kg daily) and the various concentrations of SC (1, 10 and 100 mg/kg) were administered orally to OVX + SC groups five times per week for 6 weeks¹⁴. Body weight was measured weekly from the beginning to the end of treatment.

At the end of treatment, a blood sample was collected by cardiac puncture and mice were sacrificed. Both sides of the uterus without the cervix were collected in a microtube and immediately measured. The femurs were removed for BMD and histopathology. The

experimental schedule is provided in figure 2A.

Bone histopathology

The right femurs were immediately fixed in 4% formaldehyde for 20 h and demineralized with 0.1M EDTA for 1 month for histological analysis. After demineralization, they were dehydrated with ethanol and xylene and embedded in paraffin. Sagittal sections of the capital femoral epiphysis were cut at 7 μm thickness. The cross sections of condyle were cut at a 5 μm thickness. The slides were stained with hematoxylin and eosin (H&E). Leica Application Suite (LAS) microscope software (Leica Microsystems, Buffalo Grove, IL, USA) was used to obtain digital images. The magnifications used were $\times 40$ and $\times 200$.

Measurement of bone mineral density

The left femur was collected along with attached muscles and connective tissue under aseptic conditions, and the epiphyses were excised. BMD of the left femur was determined by dual-energy X-ray absorptiometry with a PIXImus instrument (Lunar, Madison, WI).

Serum analysis

Blood samples were centrifuged at 14,000 rpm for 30 min, after which the serum was stored at -80°C until use. Serum estradiol and osteocalcin levels were estimated with Mouse/Rat estradiol ELISA kit (Calbiotech, Spring Valley, CA, USA) and Mouse Osteocalcin high sensitive EIA kit (TaKaRa, Otsu, Japan), respectively, according to the manufacturer's instructions. The concentrations of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN) and creatinine were measured using commercially available kits (NeoDIN Medical Institute, Seoul, Republic of Korea).

Cell culture

MCF-7 cells (breast cancer positive cell line) were grown in Roswell Park Memorial Institute (RPMI) 1640 medium with 10% fetal bovine serum and antibiotics (100 units/ml penicillin G and 0.1 mg/ml streptomycin; Gibco-BRL, Grand Island, NY, USA) at 37°C in an atmosphere containing 5% CO_2 that 95% humidity. All cells were passaged no more than 15 times, after which they were discarded. MCF-7 cells were seeded in 3.5-cm petri dishes (1×10^5 cells/well). 10 nmol/L E2, 100 nmol/L ICI 182, 780 (ICI) and SC extract (1, 10, and 100 $\mu\text{g}/\text{ml}$), dissolved in phenol red-free RPMI 1640 culture medium, were added and the cells were incubated for 24 h¹⁵.

Cell viability assay

The stabilized MCF-7 cells were incubated with various concentrations of SC extract (1, 10, and 100 µg/ml) dissolved in phenol red-free RPMI 1640 culture medium for 24 h. Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole (MTT) solution, and dissolution of the formazan crystals. The absorbance at 570 nm was measured using an enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, Downingtown, PA) with 10 independent samples.

Western blotting analysis

RIPA buffer (50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl) containing protease inhibitors (Roche, Hoffmann, USA) was used for protein extraction for uterine tissue and cell protein fractions. The lysate (30 µg) was denatured with 2× loading buffer and separated on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel, then electrotransferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA). Primary antibodies (Cell Signaling, USA) in TBS-T (1:1000 dilution) were incubated overnight at 4°C and secondary antibody, anti-mouse IgG, (1:2000 dilution; Cell Signaling, USA) in TBS-T, was incubated for 1 h at RT. The proteins were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Uppsala, Sweden). All samples were analyzed in triplicate.

Statistical analysis

Significance was determined by one-way analysis of variance (ANOVA) and nonparametric tests. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

Results

Identification of SC

SC was characterized with schizandrin by HPLC analysis. The retention time of schizandrin in 30% ethanol extract was 5.576 (Fig. 1). Peak on SC was synchronized with schizandrin, which is component of *S. chinensis*.

Effects of SC on uterine weights

OVX + vehicle mice gained weight after the surgery compared to normal mice. Treatment with SC inhibited body weight gain in OVX mice (Fig. 2B). In OVX + vehicle mice, the weight of the uterus decreased; however, all concentrations of SC significantly increased uterine weight (Fig.2C).

Histological changes in bone structure

Morphological and pathological changes in femur heads and condyles were observed

by H&E staining. OVX caused increases in growth plate thickness of the epiphyseal plate in sagittal section. Administration of 100 mg/kg SC to OVX mice markedly reduced the growth plate hypertrophy (Fig. 3). Femur condyles from the normal group had dense and well-formed interstitial cells filling the lateral and medial epicondyles, while pores within bone marrow cells appeared in OVX + vehicle mice. SC treatment attenuated the bone marrow pores in the lateral and medial epicondyles (Fig. 4).

Recovery of low bone mineral density and serum osteocalcin level

The effects of SC on bone mass in OVX mice were determined by measuring BMD of the left femur. BMD was significantly reduced in OVX + vehicle mice about 22.9% compared to the normal group. Similar to injection with E2, decreased BMD was significantly restored by the administration of all concentrations of SC in a dose dependent manner (21.7, 26.16 and 26.63%, respectively). Especially, BMD was restored almost up to normal levels in OVX + 100 mg/kg SC mice (Fig. 5A).

Ovariectomy significantly decreased serum osteocalcin concentration compared with normal mice. Decreased serum osteocalcin level was restored by SC treatment in a dose dependent manner. 10 and 100 mg/kg SC treatments significantly increased 18.8 and 55.3% of serum osteocalcin concentration compared to OVX + vehicle (Fig. 5B). In particular, 100 mg/kg SC treatment elevated serum osteocalcin level higher than normal group as well as E2-treated group.

Phytoestrogenic effects of SC

Serum estradiol concentration in the OVX + vehicle group was significantly lower than in the normal group. In the SC-treated groups, serum estradiol concentration was significantly increased (Fig. 6A). To examine the estrogenic effect of SC, we measured the expression of ER- α and - β in OVX-operated uterus and in MCF-7 breast cancer cells. ER- α and - β expression in the uterus was increased by SC treatment (Fig. 6B). In addition, when the cells were treated with various concentrations of SC, the expression of ER- α and - β were significantly increased (Fig. 6C).

Inhibitory effects of SC on oncogene expressions and tissue damage

We further confirmed that administration of SC did not result in oncogene expression and tissue damage. Expression of two oncogenes, c-fos and c-Jun, were not increased by treatment with SC, while they were significantly increased in the uterus of the OVX + E2 group (Fig. 7A). Similarly, a significant decrease in c-fos and c-Jun protein levels was observed between the control and SC extract-treated MCF-7 cells (Fig. 7B). Oncogene expressions such as c-fos and c-Jun were down-regulated in both of *in vivo* and *in vitro*. And SC treatment appears to have a potential cytotoxic effect on breast cancer cells by reducing cell viability at 10 and 100 $\mu\text{g/ml}$ concentrations (Fig. 7C).

The effects of SC on the liver and kidney were determined by measurement of serum AST and ALT levels, and BUN and creatinine levels, respectively. The OVX + SC group

exhibited no change in serum AST and ALT levels or BUN and creatinine levels (data not shown).

Discussion

Hormones secreted by the ovaries are disrupted by OVX surgery and the endometrium atrophies, resulting in a reduction in uterine weight¹⁶. In our experiment, ovariectomy was performed to induce post-menopausal osteoporosis. OVX + vehicle mice showed an increase in body weight and a decrease in uterine weight. The administration of SC to OVX mice resulted in recovery of uterine weight. In addition, serum estradiol levels were increased in the OVX + SC group at all concentrations. These results demonstrate that SC might have phytoestrogenic effects. This was confirmed by the expression of ER- α and - β . Estrogen binds to ER- α and - β , which are ligand-dependent transcriptional regulators, and so exerts its effects¹⁷. Generally, the expressions of ER- α and - β are active in various clinical areas such as hormone replacement, autoimmune diseases, prostate and breast cancer, and depression by appearing tissue-specific roles¹⁸. SC treatment increased the expression of ER- α and - β in the uterus similar to those in the OVX + E2 group. In addition, the protein expression of ER- α and - β in MCF-7 cells, human breast cancer which express ER was also increased by SC treatment.

Estrogen deficiency-induced osteoporosis is characterized by low bone density and microarchitectural deterioration, with the bone becoming progressively susceptible to fractures due to fragility, including in the neck of the femur, vertebrae and Colles fractures of the wrist^{19,20}. It has been shown that OVX surgery induces wider growth plates and low

density of bone marrow cells due to estrogen-mediated growth cessation²¹. E2 treatment accelerated a decrease of growth plate hypertrophy and an increase of well-formed interstitial cells. We investigated the phytoestrogenic effect of SC on BMD and femur structure. BMD levels were significantly restored by SC treatment. Histological analysis showed that SC inhibited hypertrophy of growth plate in the epiphyses of femoral head. In addition, SC treatment decreased the incidences of pores within the bone marrow cells of the lateral and medial epicondyles. To understand the further mechanism through which SC improves bone structure and BMD levels, we analyzed serum osteocalcin levels. Osteocalcin is one of the major factors in bone formation, due to its expression by osteoblasts during differentiation⁶. When the bone matrix is mineralized, osteocalcin expression is concurrently increased²². In our experiment, serum osteocalcin levels, as markers of bone turnover, were significantly increased by SC treatment. These results suggest that treatment with SC promotes bone formation by enhancing osteoblast differentiation to maturation through its phytoestrogenic effect.

Long-term treatment with the steroid hormone E2 has been known to induce the transcription of the proto-oncogenes, c-fos and c-Jun^{23, 24}. Overexpression of the two transcription factor complex, Fos-Jun heterodimeric activating protein-1, activates growth factor signaling and promotes hormone-independent growth in breast tumors²⁵. To determine the risk of cancer development, the expression of two proto-oncogenes and cell viability in MCF-7 breast cancer cells were examined. SC didn't increase the expression of c-fos and c-Jun in the uterus and breast cancer cells, while the transcription of both factors were increased after treatment with E2. In addition, proliferation of cancer cells was reduced by SC treatment.

Conclusion

Taken together, our data suggest that SC improved estrogen-deficient osteoporosis via the modulation of differentiation markers and bone structure. In addition, SC showed phytoestrogenic effects with a relatively low risk of cancer development. Consequently, SC may serve as a supplementary ingredient for menopausal females.

Acknowledgements

This research was supported by High Value-added Food Technology Development Program, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.

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Figure legends

Fig. 1. Standardization of SC (A) and standard compound (B) using high-performance liquid chromatography (HPLC) systems. Schizandrin, which is main component of *Schizandra chinensis*, were detected in SC extract.

Fig. 2. Study design (A). The effects of SC on body weight (B), and uterine weight/body weight (C) in OVX mice. Body weight were measured weekly after the treatment. After sacrifice, the uterine weight were calculated. Results are presented as mean \pm S.D. # and ## indicates the mean differs significantly between normal group and OVX + vehicle group ($p < 0.05$ and $p < 0.01$, respectively). *, ** and indicates that the mean differs significantly between OVX + SC or E2 group and OVX + vehicle group ($p < 0.05$ and $p < 0.01$, respectively).

Fig. 3. The effects of SC on growth plate thickness of the capital femoral epiphysis. The sagittal sections were stained with hematoxylin and eosin (H&E) and magnification was $\times 40$ and $\times 200$.

Fig. 4. The effects of SC on pores within interstitial cells filling the lateral and medial epicondyles. The cross sections were stained with hematoxylin and eosin (H&E) and magnification was $\times 40$ and $\times 200$.

Fig. 5. The effects of SC on bone mineral density (BMD) (A) and serum osteocalcin concentrations (B). Results are presented as mean \pm S.D. # and ### indicates the mean differs significantly between normal group and OVX + vehicle group ($p < 0.05$ and $p < 0.001$, respectively). *, ** and *** indicates that the mean differs significantly between OVX + SC or E2 group and OVX + vehicle group ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively).

Fig. 6. The effects of SC on serum estradiol concentrations (A) and the expressions of estrogen receptor- α and - β in uterus (B) and in MCF-7 breast cancer cells (C). Results are presented as mean \pm S.D. ## and ### indicates the mean differs significantly between normal group and OVX + vehicle group ($p < 0.01$ and < 0.001 , respectively). *, ** and *** indicates that the mean differs significantly between OVX + SC or E2 group and OVX + vehicle group ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively). The effects of SC on the expressions of estrogen receptor- α and - β in MCF-7 breast cancer cells (C). *** indicates that the mean differs significantly between non-treated group and E2 or SC-treated group ($p < 0.001$).

Fig. 7. The effects of SC on the expressions of c-fos and c-Jun in uterus (A). Results are presented as mean \pm S.D. ### indicates the mean differs significantly between normal group and OVX + E2 group ($p < 0.001$). ** and *** indicates that the mean differs significantly between OVX + E2 group and OVX + SC group ($p < 0.01$ and < 0.001 , respectively). The effects of SC on the expressions of c-fos and c-Jun in MCF-7 breast cancer cells (B). # and ### indicates the mean differs significantly between non-treated group and E2-treated group ($p < 0.05$ and < 0.001 , respectively). ** and *** indicates that the mean differs significantly between E2-treated group and SC-treated group ($p < 0.01$ and < 0.001 , respectively). The

effects of SC on the cell viability in MCF-7 breast cancer cells (C). * and *** indicates that the mean differs significantly between non-treated group and SC-treated group ($p < 0.05$ and < 0.001 , respectively).

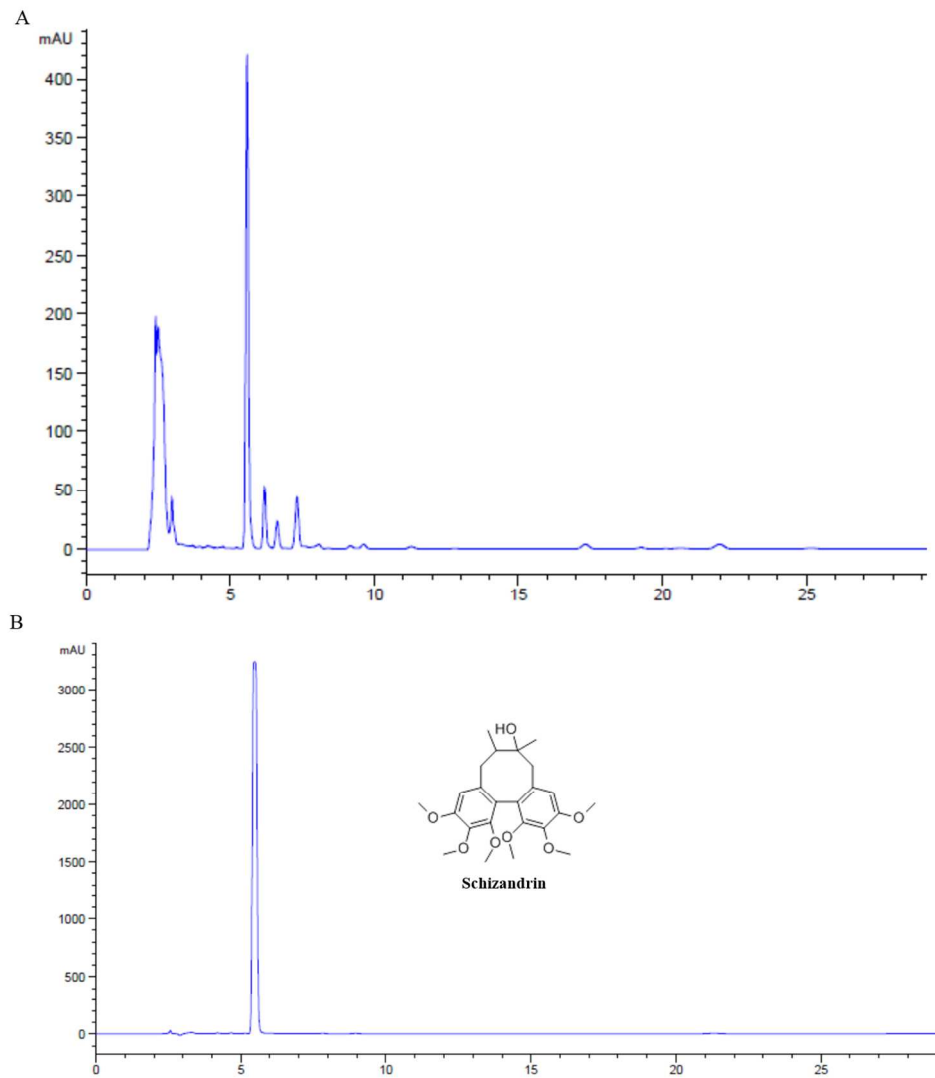


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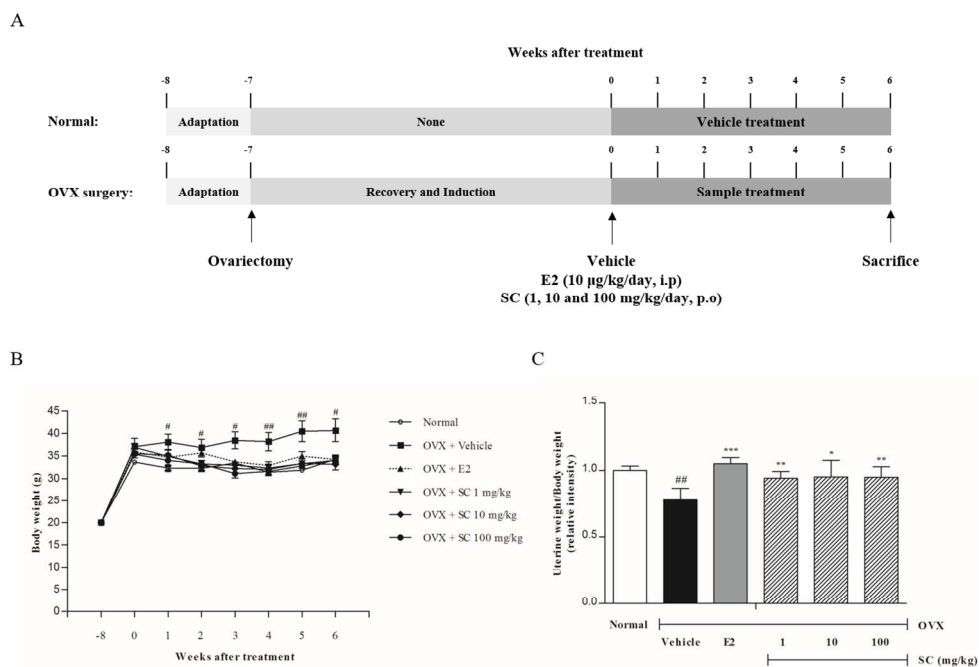


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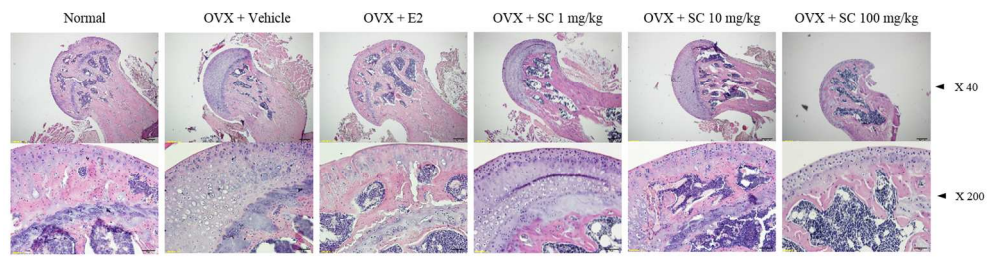


Fig. 3. The effects of SC on growth plate thickness of the capital femoral epiphysis. The sagittal sections were stained with hematoxylin and eosin (H&E) and magnification was $\times 40$ and $\times 200$.
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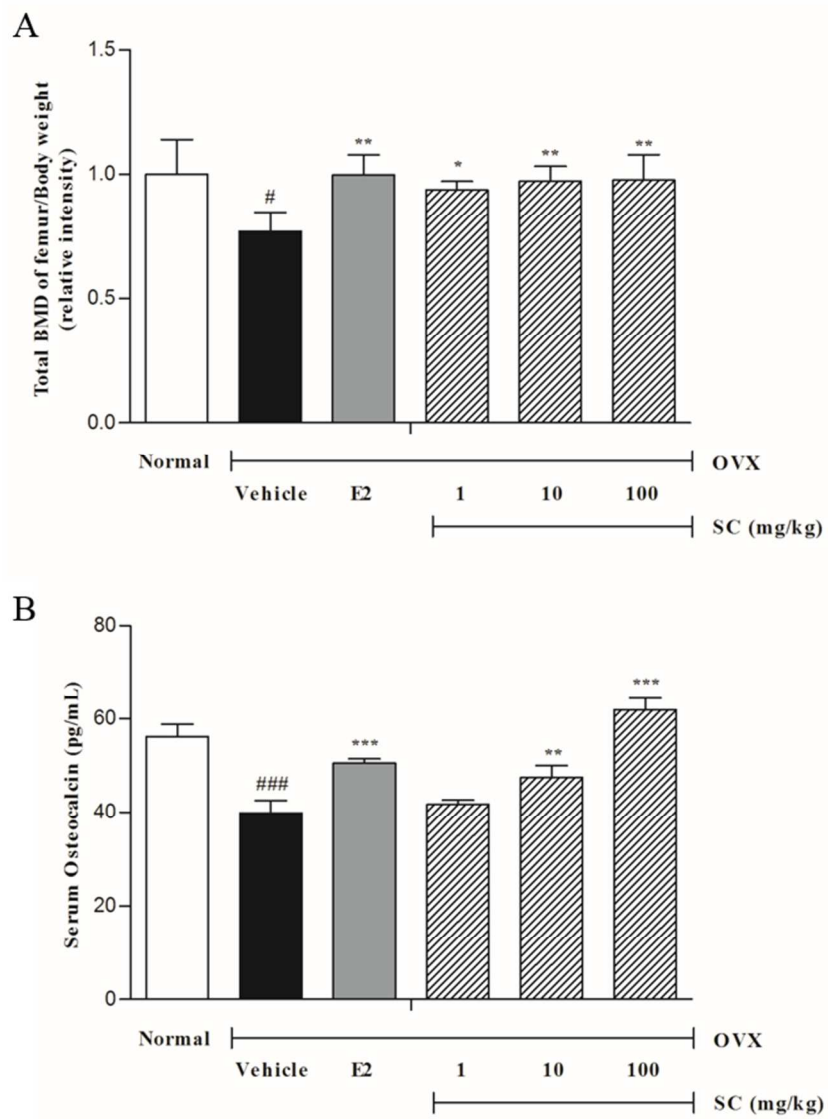


Fig. 4. The effects of SC on pores within interstitial cells filling the lateral and medial epicondyles. The cross sections were stained with hematoxylin and eosin (H&E) and magnification was $\times 40$ and $\times 200$. 119x162mm (150 x 150 DPI)

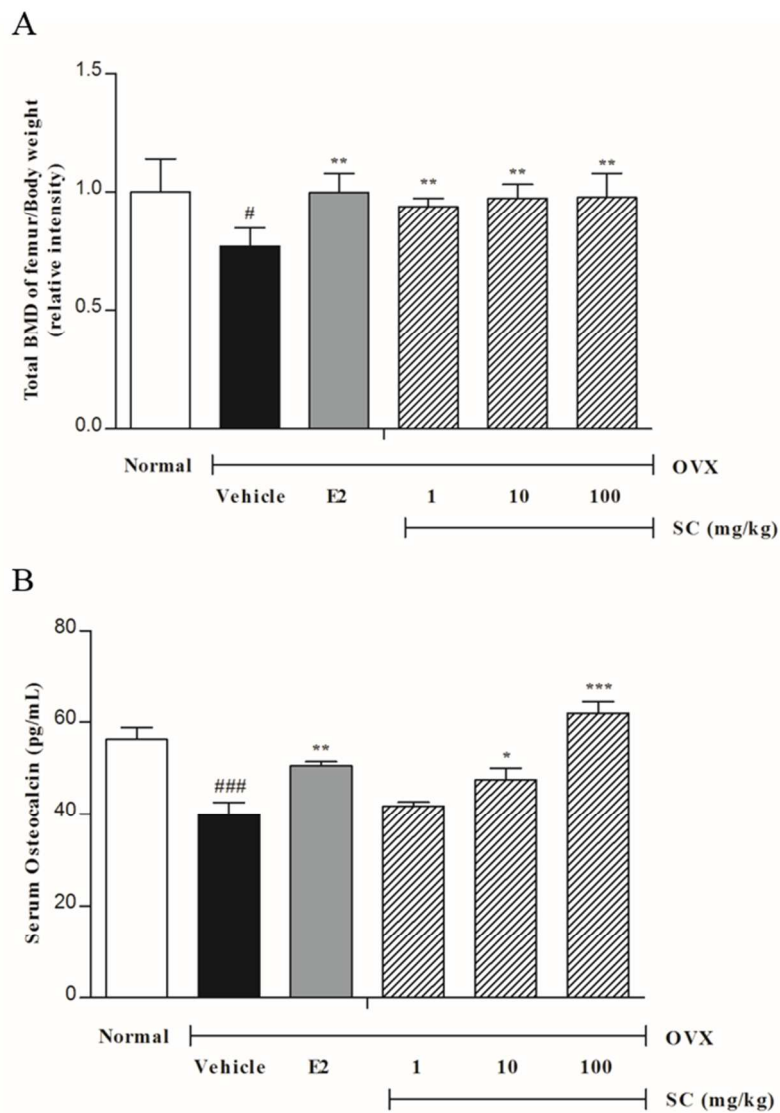


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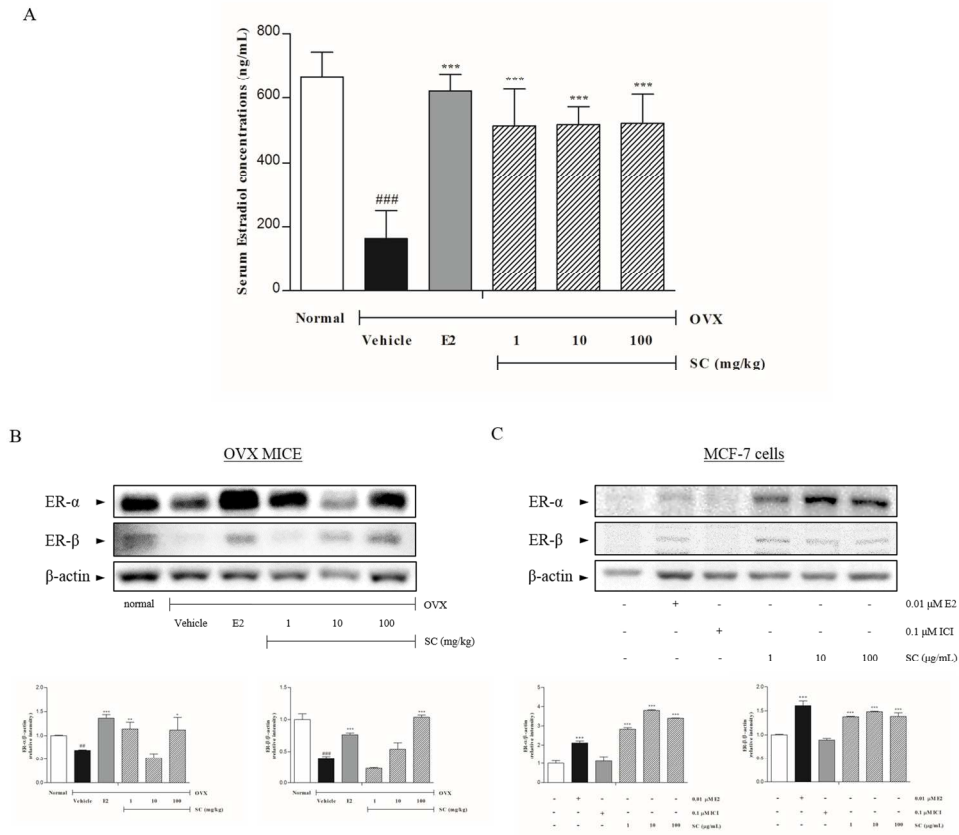


Fig. 6. The effects of SC on serum estradiol concentrations (A) and the expressions of estrogen receptor- α and - β in uterus (B) and in MCF-7 breast cancer cells (C). Results are presented as mean \pm S.D. ## and ### indicates the mean differs significantly between normal group and OVX + vehicle group ($p < 0.01$ and < 0.001 , respectively). *, ** and *** indicates that the mean differs significantly between OVX + SC or E2 group and OVX + vehicle group ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively). The effects of SC on the expressions of estrogen receptor- α and - β in MCF-7 breast cancer cells (C). *** indicates that the mean differs significantly between non-treated group and E2 or SC-treated group ($p < 0.001$).

265x231mm (300 x 300 DPI)

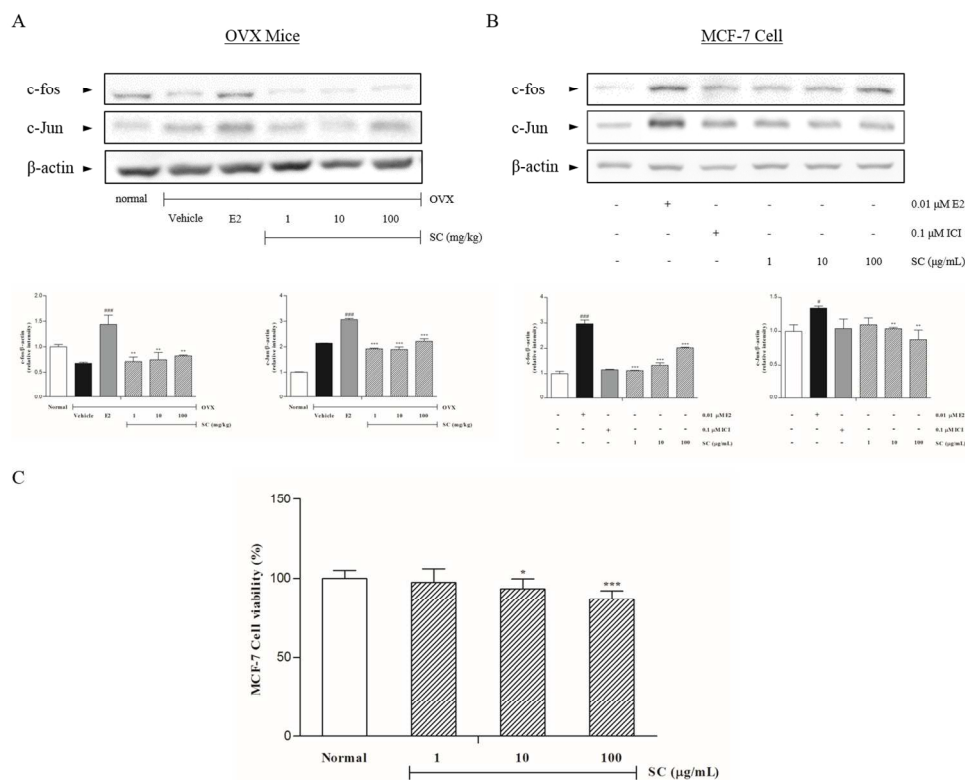


Fig. 7. The effects of SC on the expressions of c-fos and c-Jun in uterus (A). Results are presented as mean \pm S.D. ### indicates the mean differs significantly between normal group and OVX + E2 group ($p < 0.001$). ** and *** indicates that the mean differs significantly between OVX + E2 group and OVX + SC group ($p < 0.01$ and < 0.001 , respectively). The effects of SC on the expressions of c-fos and c-Jun in MCF-7 breast cancer cells (B). # and ### indicates the mean differs significantly between non-treated group and E2-treated group ($p < 0.05$ and < 0.001 , respectively). ** and *** indicates that the mean differs significantly between E2-treated group and SC-treated group ($p < 0.01$ and < 0.001 , respectively). The effects of SC on the cell viability in MCF-7 breast cancer cells (C). * and *** indicates that the mean differs significantly between non-treated group and SC-treated group ($p < 0.05$ and < 0.001 , respectively).

253x206mm (150 x 150 DPI)