

Dioscin improves postmenopausal osteoporosis through inducing bone formation and inhibiting apoptosis in ovariectomized rats

Shan Wu^{1,§}, Fan Zhao^{2,§}, Jing Zhao¹, Hui Li³, Junyu Chen¹, Yang Xia¹, Junwei Wang¹, Benzheng Zhao¹, Shuhua Zhao^{1,*}, Na Li⁴

¹The Second Hospital of Jilin University, Changchun, Jilin province, China;

²China-Japan Union Hospital of Jilin University, Changchun, Jilin province, China;

³Qian Wei Hospital of Jilin Province, Changchun, Jilin province, China;

⁴Jilin Ginseng Academic, Changchun University of Chinese Medicine, Changchun, Jilin province, China.

Summary

Postmenopausal osteoporosis (PMO) has become a public health problem worldwide. Hormonal replacement therapy (HRT) is the most popular treatment for PMO at present, but the side effects, including increased risk of endometrial cancer and breast cancer, limit its clinical use. Therefore, finding a new medication with high efficiency and less side-effects is urgently required. Dioscin is the main ingredient of some medicinal plants such as *Dioscorea nipponica* Makino and *Dioscorea zingiberensis* Wrig. It is reported that dioscin has anti-tumoral and anti-atherosclerotic activity as well as an inhibitory effect on hepatic fibrosis. In this study, the effects of dioscin on PMO were examined and the mechanisms were analyzed. The results indicated that the bone mineral density and ultimate load of PMO rats were increased after being treated with dioscin. H&E staining and immunohistochemical staining showed the bone trabeculae formation and bone differentiation of PMO rats were promoted by dioscin. Western blots revealed that dioscin could activate the PI3K/P38/AKT signaling pathway and inhibit the apoptosis signaling pathway in bone tissue cells of PMO rats. In addition, MTT assays showed that MC3T3-E1 cell viability could be improved by dioscin. These results suggest dioscin is a potential therapeutic reagent for osteoporosis and deserves further investigation.

Keywords: Dioscin, postmenopausal osteoporosis, PI3K/P38/AKT pathway, apoptosis

1. Introduction

Osteoporosis is a prevalent systemic metabolic bone disease characterized by decreased bone mass, bone microstructure degradation, bone fragility and susceptibility to pathological fractures (1). Osteoporosis has been paid more attention, because of the progressive aging of society (2). According to a report from World Health Organization, osteoporosis has become a global

health issue with high morbidity and mortality, similar to cardiovascular diseases, diabetes, and cancer. More than 200 million people in the world have osteoporosis, and at least 9 million of them suffer from fractures. The financial burden of fractures has been increasing and is expected to rise 29% by 2020 compared to 2005 (3).

There are numerous issues that can cause osteoporosis, such as endocrine or nutritional factors, age, and a myriad of chronic systemic diseases. It can occur at any age but is especially frequent in postmenopausal women (4). Postmenopausal women suffer from osteoporosis primarily due to decreased gonadal function, which, in conjunction with advanced age, promotes postmenopausal osteoporosis (PMO) (5). Both the pain and skeletal deformation caused by PMO decrease the quality of life of female patients. Moreover, PMO sufferers place a financial burden on both families and society due to the disabilities

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[§]These authors contributed equally to this work.

*Address correspondence to:

Dr. Shuhua Zhao, Department of Gynecology and Obstetrics, the Second Hospital of Jilin University, Changchun 130041, Jilin province, China.

E-mail: zhaoshuhua-1966@163.com

Table 1. The pharmacological treatment of rats

Treatments	Model group	Low dose group	High dose group	Control group
Dioscin	/	150 mg/kg daily (<i>i.g.</i>)	300 mg/kg daily (<i>i.g.</i>)	/
Operations	Ovariectomy	Ovariectomy	Ovariectomy	Sham-operation

associated with PMO. Therefore, PMO is a serious problem that still needs to be adequately addressed. It is necessary to find new remedies for the disease that will be both effective and safe, to complete the existing therapeutic arsenal.

Estrogen (E) deficiency caused by ovarian dysfunction is the major factor in the development of osteoporosis after menopause (6,7), and hormone replacement therapy (HRT) has been applied widely in recent decades. However, long term HRT is associated with an increased risk of thrombosis, breast cancer, and endometrial cancer, which limits the clinical application of HRT. Furthermore, HRT-induced sodium and water retention affect patient compliance. Apart from HRT, other treatments for PMO include calcitonin, bisphosphonates, statins, and monoclonal antibodies. However, the clinical applications are all restricted by high price and side effects, including osteonecrosis and venous thromboembolism (VTE) (8). Considering these problems, herbal drugs, which might be cable of assuaging concerns about treatment risks, have received attention in PMO management and are of great interest to both public and medical professionals.

Dioscin is the main ingredient of some medicinal plants such as *Dioscorea nipponica* Makino and *Dioscorea zingiberensis* Wright (9) and has therapeutic uses in various diseases. It is reported that dioscin has anti-tumoral activity (10,11), anti-atherosclerotic activity (12) and inhibitory effects on hepatic fibrosis (13). In recent years, several studies have showed dioscin alleviates the impact of osteoporosis (4). Interestingly, it does not cause endometrium proliferation (3), which indicates that dioscin could be used in women with intact uteri.

In this study, osteoporotic ovariectomized rat model, as well as a cultured osteoblast precursor cell line, was made to investigate the effects of dioscin on PMO. Moreover, possible molecular mechanisms of action were proposed to further verify the feasibility of clinical application of dioscin.

2. Materials and Methods

2.1. Materials

Dioscin (purity > 98%) was purchased from Shanxi Hengcheng Pharmaceutical company. In cell experiments, dioscin was added into phosphate buffered saline (PBS) with a final concentration of less than 0.1% fetal bovine serum (FBS) and penicillin-streptomycin were obtained from Hyclone. Dimethyl sulfoxide (DMSO) was

purchased from Amresco. Alkaline phosphatase (ALP), bone morphogenetic protein (BMP), PI3K, P38, p-P38, ERK, p-ERK, AKT, p-AKT, Bcl-2, Bax, Caspase3 and c-Caspase3 antibodies were purchased from Affinity.

2.2. Animal preparation

Forty female Sprague Dawley (SD) rats were provided by Basic Medical College of Jilin University. The animal study was conducted following internationally recognized guidelines and was approved by the Animal Research Committee of Norman Bethune College of Medicine, Jilin University. Apart from this, all rats were allowed seven days to adapt to the new environment and were housed in a room with a 12-hour light/dark cycle at a temperature between 22-25°C and controlled humidity between 40-50%.

2.3. Pharmacological treatment

As shown in Table 1, the study was performed on 40 female SD rats (200 ± 20 g). At about 4 to 6 weeks of age, the rats were randomly divided into four groups (of 10 animals each): Model group and control group received PBS intragastrically (*i.g.*) at a rate of 4 mL/kg daily. The other two groups were treated *i.g.* with dioscin at either 150 mg/kg daily (low dose group) or 300 mg/kg daily (high dose group) in PBS.

With the exception of the control group (which underwent the same procedure but without ovariectomy), the other 30 rats all underwent a complete bilateral ovariectomy. Five days after the operation, dioscin was administered *i.g.* for 60 days.

After intraperitoneal injection of chloral hydrate (350 mg/kg), the rats' serum was collected and used for culturing MC3T3-E1 cells. Then, the rats were euthanized, and the right femurs were dissected, cleaned, and fixed in 10% buffered formalin for 24 hrs. After that, the bones were kept in 70% ethanol and stored at 4°C to be sliced.

2.4. BMD measurement

Before euthanizing the rats, the bone mineral density (BMD) of femur and lumbar vertebra were measured via Dual-energy X-ray (LUNAR, USA) absorptiometry using software for small animals.

2.5. Three-point bending test

The mechanical properties of the femoral diaphysis

were studied using a three-point bending test. After being placed at room temperature for 1h, the left femurs were placed under vertical load in the testing machine. The load increased at the rate of 2 mm/min until the bone broke, and the load under which the bone broke (the ultimate load) was recorded.

2.6. Histomorphological assay

The right femurs of the rats were fixed in 10% buffered formalin for 24 hrs and then decalcified in 10% ethylenediaminetetraacetic acid for 14 days. The bones were then treated with ethanol and xylene for dehydration. After being embedded in paraffin and sliced, several 4 μ m-thin histological slices stained with hematoxylin and eosin (H&E) were prepared. The images were subsequently acquired using a light microscope with 200 \times magnification.

2.7. Immunohistochemical (IHC) staining

After 60 days of treatment, histological slices from the right femurs of rats were prepared for ALP and BMP IHC staining, as previously described (14). The slices were analyzed using a microscope Image-Pro Plus 6.0, and positive cells were identified as the cells with brown staining.

2.8. Western blot

The protein samples from fresh rat femoral bone tissue were extracted with cold lysis buffer, resolved in 12% SDS-PAGE gels, separated electrophoretically and transferred onto Polyvinylidene Fluoride (PVDF) membranes (Millipore, USA). The non-specific binding sites of the membrane were blocked with 5% dried skim milk at 37°C for 1hr. After that, the membrane was incubated with primary antibodies (PI3K, P38, p-P38, ERK, p-ERK, AKT, p-AKT, Bcl-2, Bax, Caspase3, c-Caspase3 and β -actin antibody) overnight at 4°C and then incubated with horseradish peroxidase (HRP) tagged secondary antibody at room temperature for 2hrs. Finally, the protein level was detected with an ECL plus kit (Millipore, USA) and developed via ChemiDoc XRS (BIO-RAD, USA).

2.9. Cell cultures and MTT assay

The MC3T3-E1 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and were cultured in IMDM supplemented with 10% FBS and 1% penicillin-streptomycin. The cells were all maintained in a humidified incubator containing 5% CO₂ at 37°C. Cell viability was evaluated with MTT assay. After being adjusted to a concentration of 5×10^4 /mL, the MC3T3-E1 cells were seeded onto 96-well plates at 100 μ L per well

and cultured in an incubator overnight. After being incubated with the serum of rats respectively for 72 hrs, the MTT solution (10 μ L per well, 5 mg/mL) was added into each well, and the cells were incubated at 37°C for 4 hrs. Finally, DMSO solution was added at 150 μ L per well. After shaking for 10 minutes, the absorbance was analyzed at 490 nm using a Microplate Reader.

2.10. Statistical analysis

All data was analyzed with the statistical software SPSS 18.0 and the results expressed as means \pm SD. The differences among groups were analyzed using a student's *t*-test. $p < 0.05$ and $p < 0.01$ indicated statistically significant differences.

3. Results

3.1. Dioscin increased the BMD of PMO rats

To assess the effect of dioscin on PMO, the BMD of PMO rats were measured. As shown in Figure 1A-C, rats in the model group showed a lower level of femur BMD (f-BMD) and lumbar vertebra BMD (v-BMD) compared with the control group ($p < 0.01$). Meanwhile, low-dose treatment with dioscin increased the level of f-BMD and v-BMD ($p < 0.01$) compared with the model group. Furthermore, high-dose treatment with dioscin increased the level of BMDs significantly ($p < 0.05$ in both BMDs). These results indicate that the PMO rat model has been established successfully, and dioscin can increase the BMD of PMO rats by promoting formation of bone.

3.2. Dioscin enhanced the mechanical properties of bone in PMO rats

To further investigate whether dioscin improves mechanical properties of bone in the PMO rats, maximum bone stress was compared between groups. As shown in Figure 1D, the femurs from the model group show a significant decrease in ultimate load compared with the control group ($p < 0.01$), and a substantial increase in the ultimate load has been observed in both the low-dose group ($p < 0.05$) and high-dose group ($p < 0.01$) compared with the model group, suggesting dioscin is capable of providing significant protection against ovariectomy-induced bone fragility.

3.3. Dioscin increased bone trabeculae formation in PMO rats

H&E staining was employed to observe the morphological features of bone trabeculae in PMO rats. Compared with the control group, there were more spaces within the bone marrow than bone trabeculae in the cancellous bone of femurs in the model group,

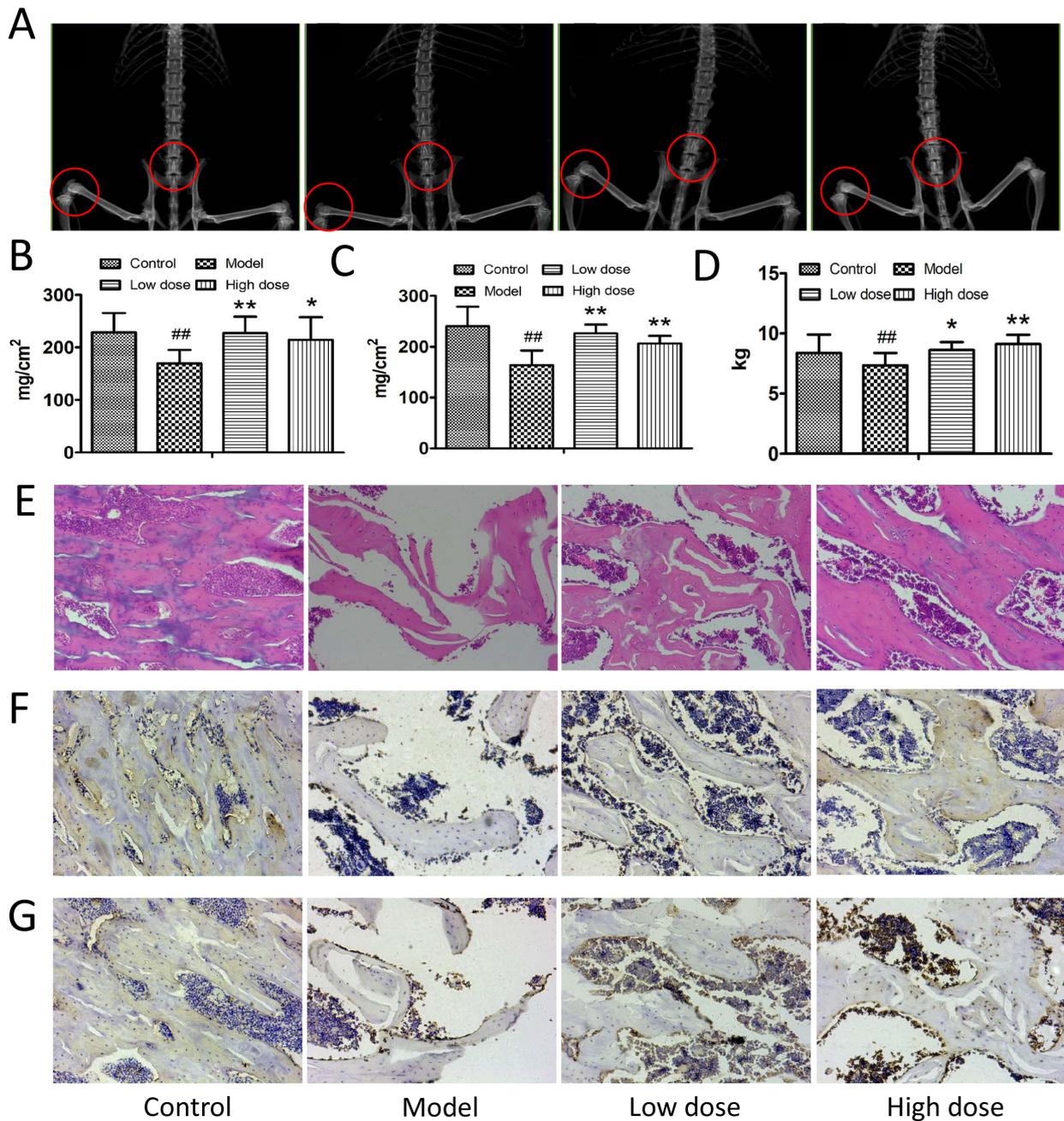


Figure 1. Effects of dioscin on BMD, ultimate load and histopathological evaluation in rats. (A-C), Effects of dioscin on the BMD of femur (A,B) and lumbar vertebra (A,C), respectively. (D), Effects of dioscin on the ultimate load of rat femur. (E), H&E staining of rat femur tissue. (F), IHC staining of ALP in rat femur tissue. (G), The IHC staining of BMP in rat femur tissue. Results were obtained from three independent experiments and are expressed as mean ± SD. #: $p < 0.05$; ##: $p < 0.01$ vs. control group; *: $p < 0.05$; **: $p < 0.01$ vs. model group.

which could be significantly restored by dioscin regardless of dose (Figure 1E). These results demonstrate the protective role of dioscin in the prevention of ovariectomy-induced trabecular bone loss.

3.4. Dioscin promoted bone differentiation

To further verify the efficacy of dioscin in the promotion of bone differentiation, IHC staining was conducted to determine the levels of ALP and BMP. As shown in Figure 1F, the expression of ALP in rats from the model

group is lower than control group, while this decrease has been prevented by dioscin. The same change can be observed in the expression of BMP (Figure 1G). Therefore, the conclusion can be drawn that dioscin is capable of up-regulating the levels of ALP and BMP, which have been down-regulated in PMO rats.

3.5. Dioscin promoted bone formation via PI3K/P38/AKT signaling pathway

To investigate the mechanism of dioscin promoting the

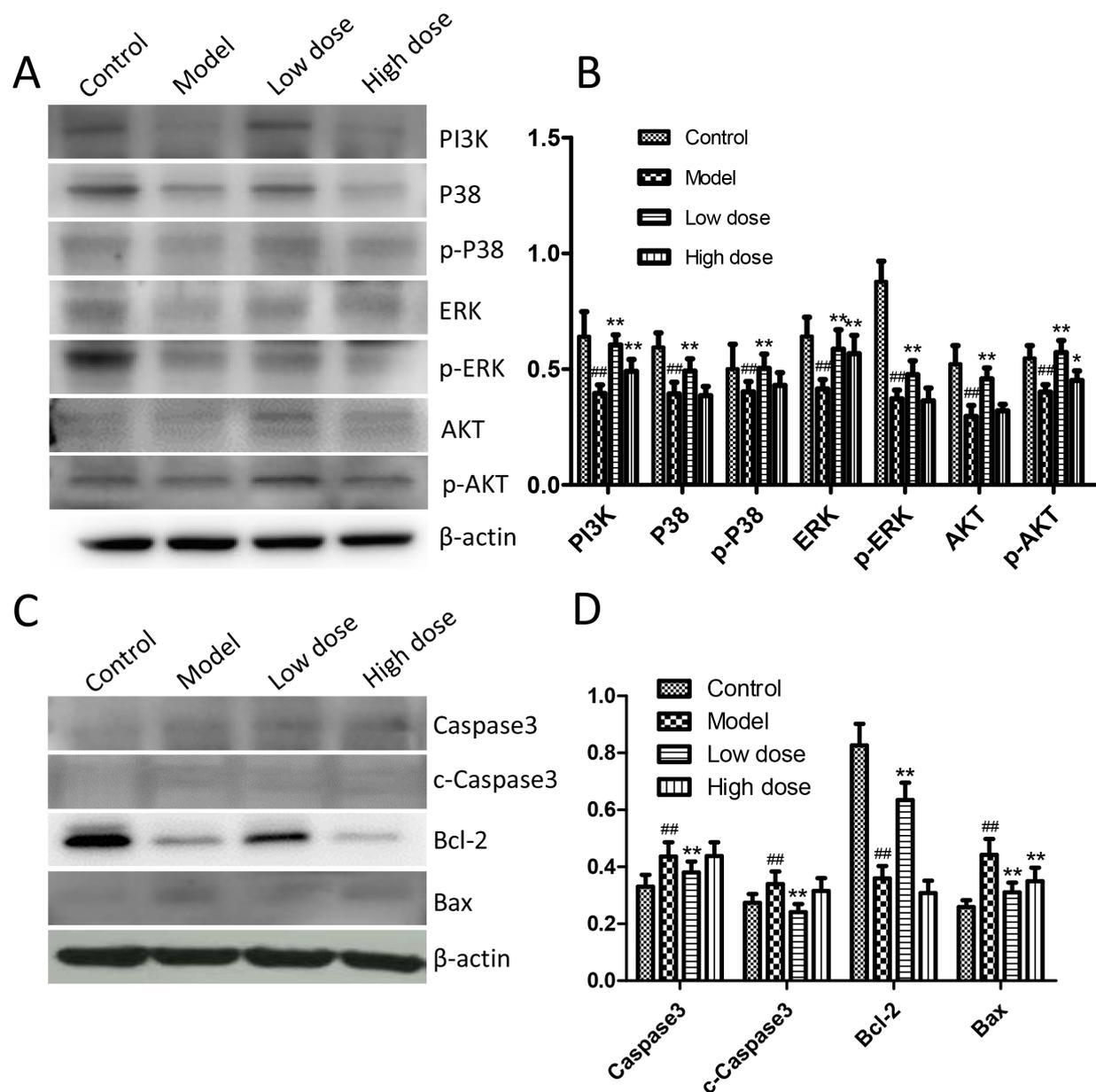


Figure 2. Effects of dioscin on proliferation, differentiation and apoptosis-associated proteins in bone tissue. (A and B), Effects of dioscin on PI3K, P38, p-P38, ERK, p-ERK, AKT and p-AKT levels in bone tissue. (C and D), Effects of dioscin on Caspase3, c-Caspase3, Bcl-2 and Bax levels in bone tissue. Results were obtained from three independent experiments and are expressed as mean \pm SD. #: $p < 0.05$; ##: $p < 0.01$ vs. control group; *: $p < 0.05$; **: $p < 0.01$ vs. model group.

formation of bone, Western blots were used to detect the expression of proliferation-associated proteins in bone tissue. As shown in Figure 2A and Figure 2B, the expression of PI3K, P38, p-P38, ERK, p-ERK, AKT, p-AKT of rats in the model group was decreased compared with the control group ($p < 0.01$). Except that the level of P38, p-P38, p-ERK and AKT was only increased by low-dose dioscin ($p < 0.01$), the increase of the other proteins were observed at both the low and high dose of dioscin, compared to the model group. These results reveal that the promoting effect of dioscin in bone formation might be associated with the activation of the PI3K/P38/AKT signaling pathway.

3.6. Dioscin inhibited the apoptosis of bone tissue

To further explore the mechanism of dioscin promoting the formation of bone, the expression of apoptosis-associated proteins in bone tissue was detected using Western blots. Results are represented in Figure 2C and Figure 2D: in the model group, the level of Bcl-2 was decreased, and the level of Caspase3, c-Caspase3 and Bax was increased compared with the control group ($p < 0.01$). While it was expected only low-dose dioscin would effectively down-regulate the level of Caspase3 and c-Caspase3, as well as up-regulate the level of Bcl-2, both dosages of dioscin inhibited the expression of

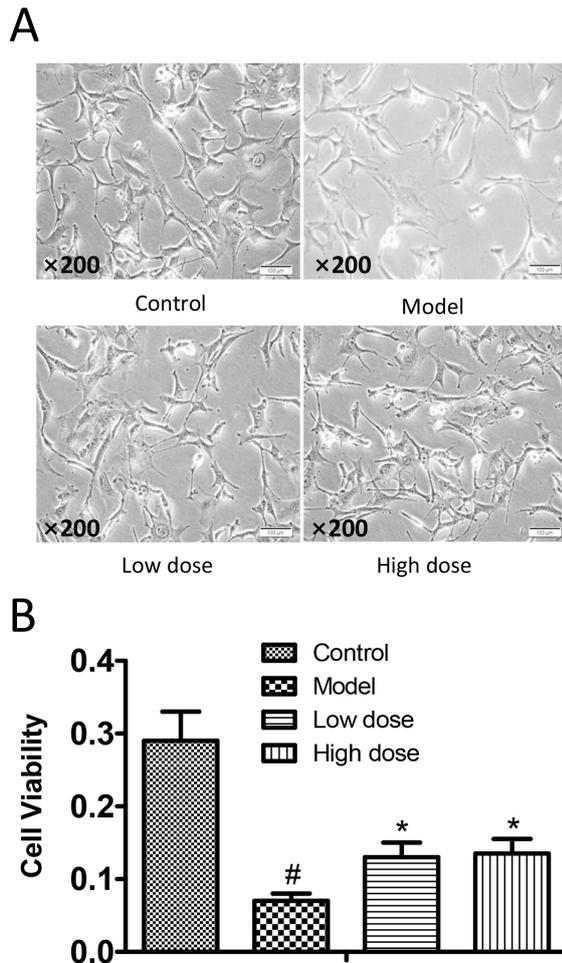


Figure 3. Effects of dioscin on the proliferation of MC3T3-E1 cells. Effects of dioscin on cell viabilities of MC3T3-E1 cells. Results are expressed as mean \pm SD. #: $p < 0.05$; #: $p < 0.01$ vs. control group; *: $p < 0.05$; **: $p < 0.01$ vs. model group.

Bax significantly ($p < 0.01$). These results suggest that dioscin could improve osteoporosis through inhibiting the apoptosis signaling pathway.

3.7. Dioscin enhanced the proliferation of MC3T3-E1 cells

MC3T3-E1 cells were used to identify whether dioscin could enhance osteoblast proliferation *in vitro*. As shown in Figure 3A and Figure 3B, the MTT assay showed that the cell viability of the model group is much lower than that of the control group. Meanwhile, the viability of cells incubated with the serum of rats treated with dioscin at different doses increased significantly, compared to the model group. The above results show that osteoblast proliferation was promoted by dioscin, which indicates dioscin might promote bone formation *via* osteoblast activation.

4. Discussion

Bone metabolic balance depends on the interaction

of bone formation and bone resorption, indicating the disorder is caused by an imbalance between osteoblasts and osteoclasts (15,16), which is also the key cause of PMO. Osteoblasts play a vital role in bone formation and are controlled by several signaling pathways, including PI3K/P38/AKT pathways (17-19). This study aimed to evaluate the osteoprotective effects of dioscin on PMO rats and explore dioscin's mechanism of action.

PMO rats, a well-established experimental model of PMO *in vivo*, were confirmed by the decrease of BMD, ultimate load, ALP and BMP expression (all of which were prevented by dioscin in this study). The lower levels of BMD and the reduced stiffness are two principal features in osteoporosis formation, predisposing patients with osteoporosis to a high risk of bone fracture (8). ALP, functioning as an osteoblast differentiation marker at an early stage, participates in the beginning of mineralization of bone formation. Additionally, ALP activation could be increased by BMP (20), a significant autocrine and paracrine growth factor (21).

BMP is a member of the TGF- β superfamily and is derived from osteoblasts. Besides, BMP could influence the differentiation of mesenchymal stem cells (MSCs) to osteoblasts *via* the P38 signaling pathway (22). Thus, BMP represents a critical factor in the process of bone formation and bone repair (23). Our results indicated that dioscin could up-regulate the expression of osteoblast differentiation-associated proteins including ALP and BMP, which implied that it might increase BMD *via* promoting osteoblast differentiation and hence strengthen the ultimate load of PMO rat femurs.

Obviously, there is a decrease in the expression of PI3K, P38, p-P38, ERK, p-ERK, AKT and p-AKT in PMO rats. Interestingly, although the effectiveness of dioscin varied based on the concentration, it could reverse these decreases in our study. P38 mitogen-activated protein kinase (P38) and extracellular regulated protein kinases (ERK) are members of mitogen-activated protein kinases (MAPK), which are a family of serine/threonine protein kinases. P38 is capable of increasing the proliferation of osteoblasts and inhibiting the differentiation of osteoclasts by activating NF- κ B, Max, P53, and Stat1 to improve osteoporosis (24). Moreover, P38 is an osteoblast-derived protein with a significant effect on the regulation of cell proliferation (25). Also, ERK has an essential impact on maintaining the balance between bone formation and bone resorption by promoting the differentiation of MSCs to osteoblasts. What's more, protein kinase B (AKT) is the downstream of PI3K (26). PI3K/AKT signaling pathway plays a vital role in osteoblast differentiation and mineralization (27). Not only does PI3K regulate the differentiation of osteoblasts by interacting with Runx2 (28-31), but also could inhibit osteoblast apoptosis by inactivating FoxO (forkhead proteins) (32-35). Apart from these, the PI3K/

AKT signaling pathway promotes the proliferation and differentiation of osteoblasts through associated signaling molecules such as ALP and BMP (36,37). Our results indicate that dioscin stimulates bone tissue proliferation and differentiation *via* P38 and PI3K/AKT signaling pathways.

Our study demonstrates the expression of Bcl-2 is up-regulated in dioscin-treated rats compared with PMO rats, while the expressions of Caspase3, c-Caspase3 and Bax are down-regulated. Bcl-2, Bax, and Caspase3 as well as c-Caspase3 play a vital role in apoptosis. Apoptosis could be induced by the release of cytochrome C from mitochondria (38). The release of cytochrome C could be inhibited by Bcl-2 and be promoted by Bax, then contribute significantly to activate the Caspase family of proteins, especially Caspase3 (39,40), which could be activated by Caspase9, cleaved into c-Caspase3 and work as an executor leading to cells apoptosis. Furthermore, by combining with Bax and silencing it, Bcl-2 performs a protective effect on apoptosis. However, the overexpression of Bax could overcome the protective effect of Bcl-2 and lead to apoptosis (41). AKT also has a protective effect *via* the inhibition of Caspase9 and activation of Bcl-2. Our results demonstrate the ability of dioscin to alleviate ovariectomy-induced osteoporosis by inhibiting bone tissue apoptosis through the regulation of both Bcl-2/Bax and PI3K/AKT signaling pathways.

Additionally, osteoblast-like MC3T3-E1 cells were used to examine the effects of dioscin on osteoblasts *in vitro*. Our data shows both low and high doses of dioscin significantly increase MC3T3-E1 cell viability. The ability to increase MC3T3-E1 cell viability provides further evidence for the mitigating effect of dioscin on osteoporosis *via* osteoblast proliferation.

Ultimately, it was found that dioscin can improve BMD and strengthen the maximum bone stress of PMO rats. The strengthening can likely be attributed to the activation of osteoblast proliferation and differentiation. Furthermore, dioscin could promote bone tissue proliferation through PI3K/AKT and P38 signaling pathways, and inhibit bone tissue apoptosis *via* regulation of the Bcl-2/Bax signaling pathway in rat models, to perform a protective effect against postmenopausal osteoporosis. The anti-osteoporotic effect of dioscin was also confirmed *in vitro* in this study. Accordingly, dioscin represents a novel candidate for treatment of PMO.

However, considering this study was preclinical, further research is required to support these conclusions. Moreover, the exact mechanisms and the clinical applications of dioscin in PMO treatment need to be further explored in the future.

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