# Ginsenoside Rb1 prevents steroid-induced avascular necrosis of the femoral head through the bone morphogenetic protein-2 and vascular endothelial growth factor pathway

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**Abstract.** At present, the molecular mechanism underlying the protective effect of Ginsenoside Rb1 remains unclear. The present study was designed to investigate whether Ginsenoside Rb1 weakened the steroid-induced avascular necrosis of the femoral head (SANFH) and to explore the possible mechanisms of the above effects. As a result, it was revealed that Ginsenoside Rb1 was protective against steroid-induced avascular necrosis and inhibited serum osteocalcin in a rat model of SANFH. Ginsenoside Rb1 reduced inflammation, oxidative stress and bone cell apoptosis in a rat model of SANFH. Furthermore, Ginsenoside Rb1 attenuated trabecula parameters, total cholesterol and low density lipoprotein/high density lipoprotein in SANFH rat. Additionally, Ginsenoside Rb1 significantly reversed alkaline phosphatase and osteocalcin activities, vascular endothelial growth factor (VEGF) receptor, VEGF, Runt related transcription factor 2 (Runx2) and bone morphogenetic protein (BMP)-2 protein expression in SANFH rat. Collectively, the present study demonstrated that Ginsenoside Rb1 attenuated SANFH through the VEGF/RUNX2/BMP-2 signaling pathway.

## Introduction

Steroid-induced avascular necrosis of the femoral head (SANFH) is caused by a variety of factors, including apoptosis, inflammation, reactive oxygen species and oxidative stress (1). SANFH is characterized by bone trabeculae and bone marrow necrosis (1). Cases of SANFH are increasing worldwide and patient outcomes are poor (2). Thus, developing strategies to effectively prevent and/or treat SANFH is of critical importance (2).

SANFH is defined by the interruption and impairment of blood supply to the femoral head, which triggers osteocyte and bone marrow necrosis (3). This leads to alterations in the structure of the femoral head, collapse and joint function disturbance. However, the pathogenesis of SANFH remains largely unclear (4). Clinically, femoral head necrosis can be divided into traumatic and non-traumatic types (4). The prolonged administration of hormones is a risk factor for non-traumatic femoral head necrosis (5).

Ginsenoside Rb1 is a dammarane-type triterpenoid saponin that is predominantly found in Panax plants, such as ginseng, *Panax notoginseng* and American ginseng (6). Panax plants are valuable Chinese herbal medicines with a long history of application (7). Ginsenoside Rb1 harbors a variety of pharmacological activities in the central nervous system, cardiovascular system and immune system, as well as anti-tumor, anti-hepatic ischemia-reperfusion injury, and hypoglycemic effects (7,8). Shen *et al* (9) reported that Ginsenoside Rb1 reduces fatty liver by activating adenosine monophosphate-activated protein kinase in obese rats. Wang *et al* (10) reported that Ginsenoside Rb1 inhibits free fatty acidinduced oxidative stress and inflammation in 3T3L1 adipocytes. The present study was designed to investigate whether Ginsenoside Rb1 weakened the steroid-induced avascular necrosis of the femoral head (SANFH) and to explore the possible mechanisms of the above effects.

## Materials and methods

*Animals, reagents and in vivo experiments.* Adult male Sprague-Dawley rats (230-260 g; 6-9 weeks; n=30) were housed at 24±2˚C and 55±2% humidity, with free access to food and water, and a 12-h light/dark cycle, at the Department of Laboratory Animal Science Affiliated to Southwest Medical University (Luzhou, China). All procedures were approved by the Ethical Committee of Animal Experimentation at Southwest Medical University.

Rats were randomly divided into three groups: i) Blank control group (sham; n=10); ii) model group (n=10); and iii) Ginsenoside Rb1 treatment group (n=10). Rats in the model

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and Ginsenoside Rb1 groups received intragluteal injections of 50 mg/kg dexamethasone twice per week for 6 weeks. After the induction of SANFH for 3 weeks, rats in the Ginsenoside Rb1 group received 200 mg/kg/week Ginsenoside Rb1 (Fig. 1A; Shanghai No. 1 Biochemical & Pharmaceutical Co., Ltd.) by oral gavage for 3 weeks (11). In the blank control and model groups, rats were given normal saline for 3 weeks by the same method.

*ELISA*. Following treatment with Ginsenoside Rb1, blood samples were collected under anesthesia (35 mg/kg of pentobarbital sodium) and centrifugated at 12,000 x g for 10 min at 4˚C. Serum osteocalcin (OST; cat. no. H152), total cholesterol (cat. no. A111-1-1) and the ratio of low-density lipoprotein to high-density lipoprotein (LDL/HDL; cat. no. A113-1-1 and A112-1-1) were examined using ELISA (all from Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's protocol. Absorbance was measured at 450 nm with a microplate reader (Bio-Rad Laboratories).

*Histopathological examination*. Following treatment with Ginsenoside Rb1, rats were sacrificed by decapitation under anesthesia (35 mg/kg pentobarbital sodium). Femur samples were obtained and fixed in 10% buffered neutral formalin solution for 3-4 days at room temperature and decalcified in 10% EDTA, 0.1 M phosphate buffer. Tissue was dehydrated with ethanol, embedded in paraffin and cut into 4 $\mu$m thick sections. Sections were stained with hematoxylin for 10 min at room temperature, and rinsed with running water for 15 min. Sections were then stained with eosin for 30 sec at room temperature, and double distilled water was used to wash the sections. The sections were dehydrated using 100% ethanol for 1 min at room temperature, cleared in xylene and sealed with neutral balsam. Sections were observed using a Zeiss Axioplan 2 light microscope (magnification, x10; Carl Zeiss AG).

*Inflammatory factor, oxidative stress, alkaline phosphatase (ALP), OST and caspase-3 activity analysis*. Femoral heads were washed in PBS and lysed with RIPA buffer (Beyotime Institute of Biotechnology) on ice for 1 h. The supernatant was collected by centrifugation at 12,000 x g for 10 min at 4˚C. For caspase-3 activity, samples were incubated with specific colorimetric peptide substrates (Ac-DEVD-pNA; Beyotime Institute of Biotechnology) for 1 h at 37˚C, and the absorbance was measured at 405 nm with a microplate reader (Eppendorf). ALP (cat. no. A059-2-2), OST (cat. no. H152), p65 (cat. no. H202), tumor necrosis factor (TNF)-α (cat. no. H052), interleukin (IL)-1β (cat. no. H002), IL-6 (cat. no. H007), malondialdehyde (MDA; cat. no. A003-1-2), superoxide dismutase (SOD; cat. no. A001-3-2), chloramphenicol acetyltransferase (CAT; cat. no. A007-1-1) and glutathione peroxidase (GSH-PX; cat. no. A005-1-2) expression was measured using ELISA kits (all from Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's protocol. Absorbance was measured at 450 nm with a microplate reader (Eppendorf).

*Western blot analysis*. Femoral heads were washed in PBS and lysed with RIPA buffer on ice for 1 h. The supernatant was collected by centrifugation at 12,000 x g for 10 min at 4˚C and protein concentration was determined with a bicinchoninic acid protein assay. Proteins (50 $\mu$g/lane) were separated by 8-10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% non-fat milk in TBST for 1 h at 37˚C and incubated with anti-apoptosis regulator BAX (Bax; 1:500; cat. no. sc-20067; Santa Cruz Biotechnology, Inc.), anti-cellular tumor antigen p53 (p53; 1:500; cat. no. sc-47698; Santa Cruz Biotechnology, Inc.), anti-vascular endothelial growth factor (VEGF) receptor (VEGFR; 1:2000; cat. no. 2479; Cell Signaling Technology, Inc.), anti-VEGF (1:500; cat. no. sc-7269; Santa Cruz Biotechnology, Inc.), anti-Runt-related transcription factor 2 (RUNX2; 1:500; cat. no. sc-390715; Santa Cruz Biotechnology, Ltd.), anti-bone morphogenetic protein 2 (BMP2; 1:500; cat. no. sc-137087; Santa Cruz Biotechnology, Ltd.) and anti-GAPDH (1:2,000; cat. no. sc-69778; Santa Cruz Biotechnology, Ltd.) primary antibodies at 4˚C overnight. Following washing with TBST, the membranes were incubated with a goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (1:5,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc.) for 1 h at 37˚C. Membranes were visualized using BeyoECL Moon reagent (Beyotime Institute of Biotechnology) and analyzed with Quantity One software (version 3.0; Bio-Rad Laboratories, Inc.).

*Statistical analysis*. All data were expressed as the mean ± standard deviation and analyzed using SPSS 17.0 (SPSS, Inc.). A minimum of three independent experiments were carried out and were analyzed by one-way analysis of variance and Tukey's post hoc test. using SPSS 17.0. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Ginsenoside Rb1 improves ALP and OST activities in the SANFH rat model*. To assess the effects of Ginsenoside Rb1 on ALP and OST activity in the SANFH rat model, ALP and OST activities were determined using ELISA kits. Firstly, H&E staining showed that the bone cell number was reduced in SANFH rats, compared with the control group (Fig. 1B). Ginsenoside Rb1 appeared to increase the number of bone cells, compared with the untreated rat model (Fig. 1B). Furthermore, ALP (Fig. 1C) and OST (Fig. 1D) activity in the rat model was significantly decreased, compared with the control group. Ginsenoside Rb1 administration significantly increased ALP and OST activity, compared with untreated model rats.

*Protective effects of Ginsenoside Rb1 on avascular necrosis of the SANFH rat model*. Serum OST expression was significantly decreased in the SANFH model group, compared with the control (Fig. 2A). In addition, the total cholesterol (Fig. 2B) and LDL/HDL ratio (Fig. 2C) were higher in the model group, compared with the control. Treatment with Ginsenoside Rb1 significantly elevated serum OST expression, and reduced total cholesterol and LDL/HDL ratio in SANFH rats.

*Protective effects of Ginsenoside Rb1 on inflammation of the SANFH rat model*. To assess the effects of Ginsenoside Rb1 on inflammation in SANFH, p65, TNF-α, IL-1β and IL-6 expression was determined using ELISA kits. p65 (Fig. 3A), TNF-α (Fig. 3B), IL-1β (Fig. 3C) and IL-6 (Fig. 3D) expression was

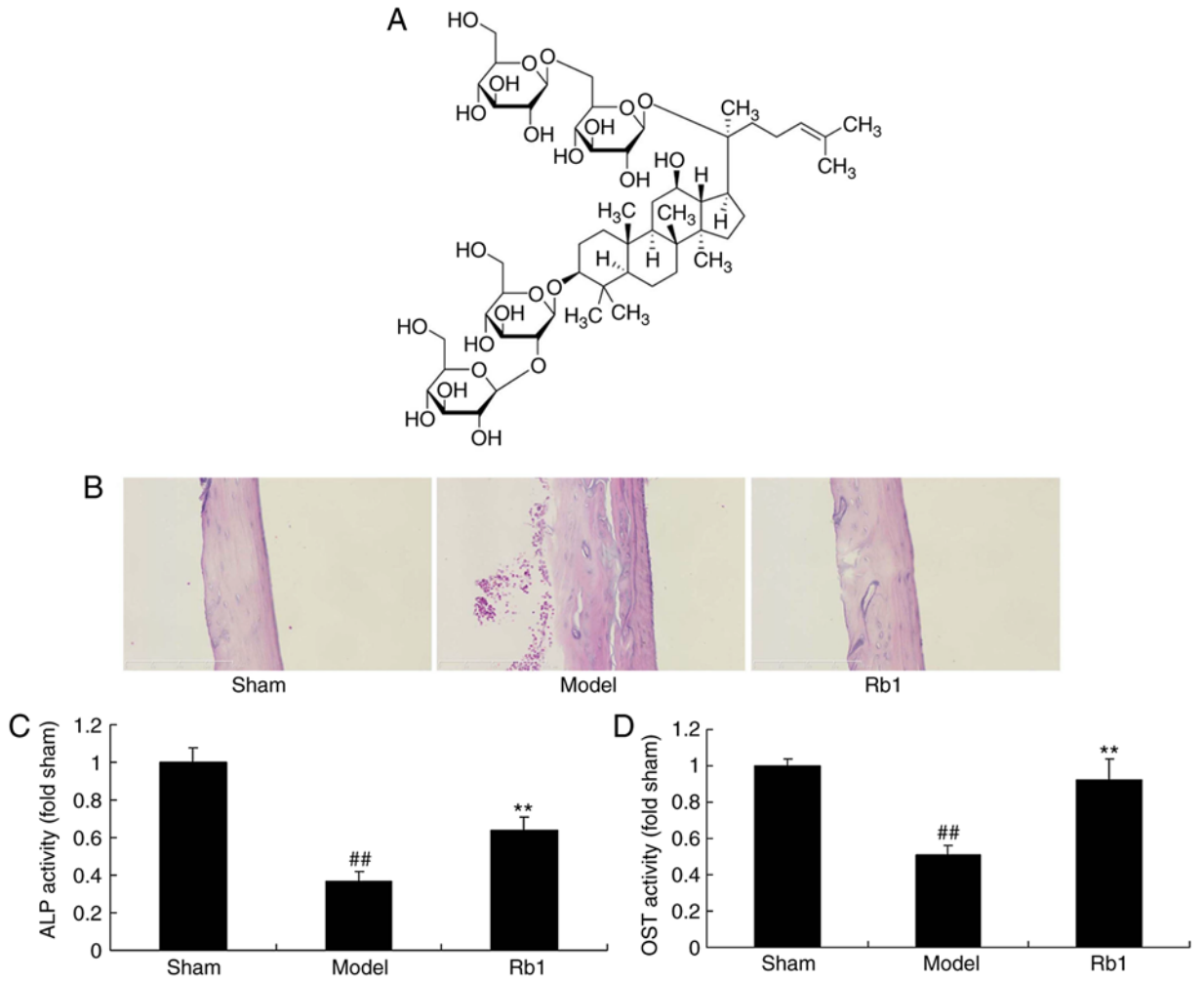


Figure 1. Protective effects of Ginsenoside Rb1 on avascular necrosis of the SANFH rat model. (A) Structural formula of Ginsenoside Rb1. (B) The SANFH rat model was evaluated by hematoxylin and eosin staining (magnification, x10), and the (C) ALP and (D) OST activities of the SANFH rat model were determined. Data are presented as the mean ± standard deviation. ##P<0.01 vs. Sham; **P<0.01 vs. Model. SANFH, steroid-induced avascular necrosis of the femoral head; Sham, sham control group; Model, SANFH model group; Rb1, 200 mg/kg of Ginsenoside Rb1 group; ALP, alkaline phosphatase; OST, osteocalcin.

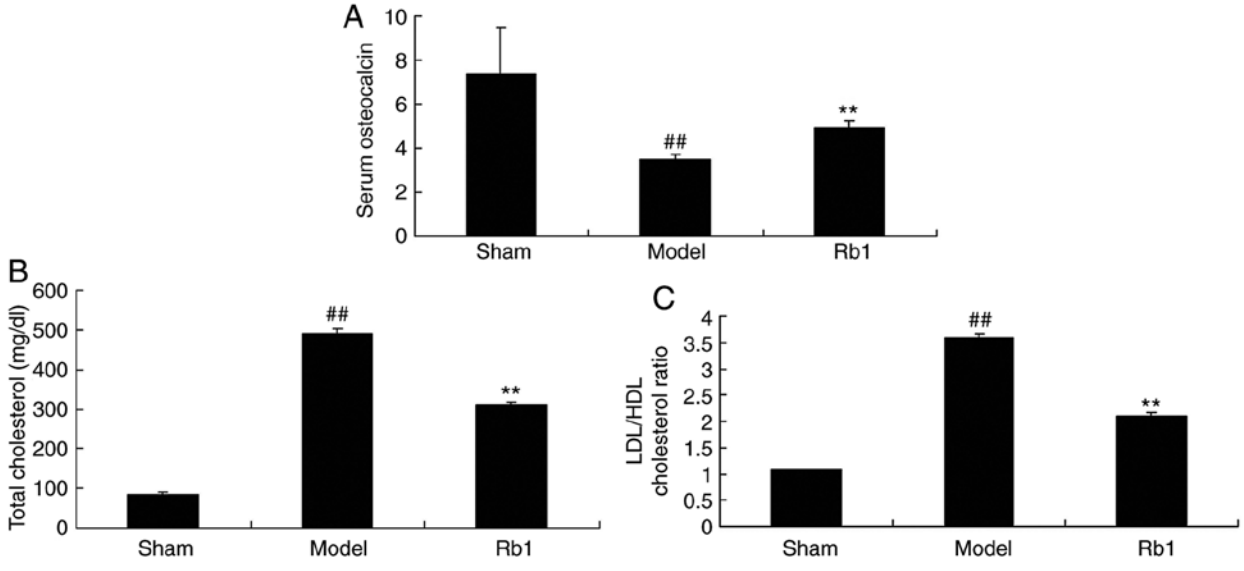


Figure 2. Protective effects of Ginsenoside Rb1 on avascular necrosis of the SANFH rat model. Protective effects of Ginsenoside Rb1 on the (A) serum osteocalcin level, (B) total cholesterol levels and (C) the LDL/HDL cholesterol ratio of the SANFH rat model. Data are presented as the mean ± standard deviation. ##P<0.01 vs. Sham; **P<0.01 vs. Model. SANFH, steroid-induced avascular necrosis of the femoral head; LDL, low density lipoprotein; HDL, high density lipoprotein; Sham, sham control group; Model, SANFH model group; Rb1, 200 mg/kg of Ginsenoside Rb1 group.

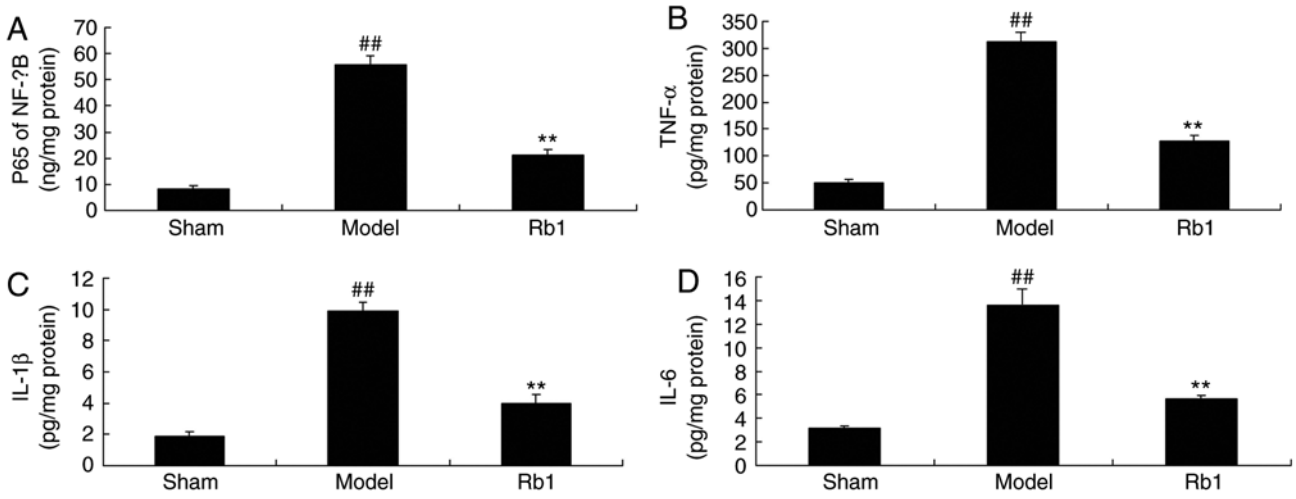


Figure 3. Protective effects of Ginsenoside Rb1 on inflammation in the SANFH rat model. Protective effects of Ginsenoside Rb1 on (A) p65, (B) TNF-α, (C) IL-1β and (D) IL-6 levels. Data are presented as the mean ± standard deviation. $^{\#\#}$P<0.01 vs. Sham; $^{**}$P<0.01 vs. Model. SANFH, steroid-induced avascular necrosis of the femoral head; Sham, sham control group; Model, SANFH model group; Rb1, 200 mg/kg of Ginsenoside Rb1 group; TNF-α, tumor necrosis factor-α; IL, interleukin.

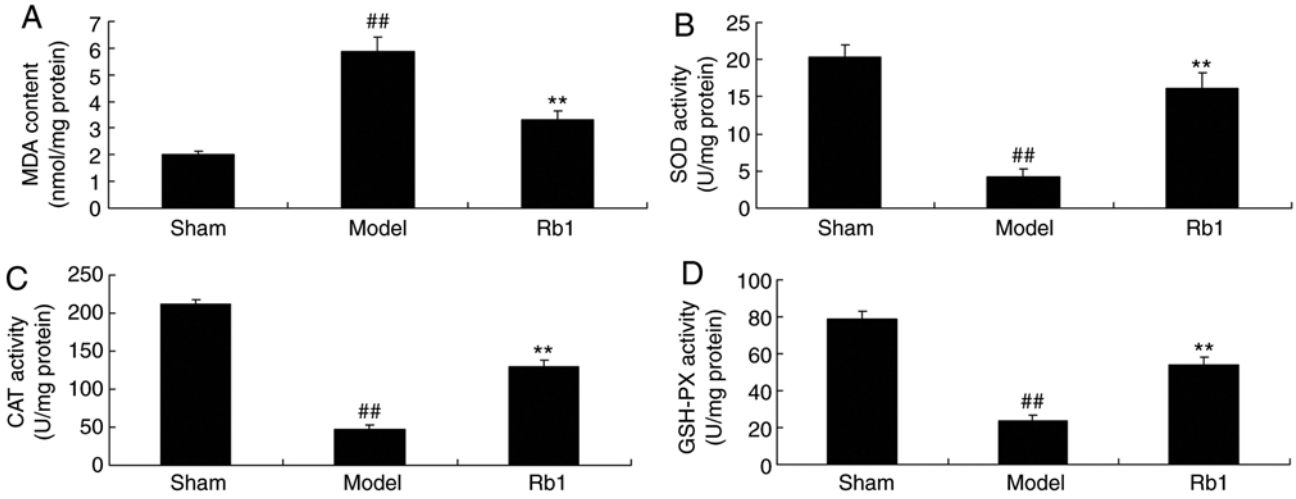


Figure 4. Protective effects of Ginsenoside Rb1 on oxidative stress in the SANFH rat model. Protective effects of Ginsenoside Rb1 on (A) MDA, (B) SOD, (C) CAT and (D) GSH-PX. Data are presented as the mean ± standard deviation. $^{\#\#}$P<0.01 vs. Sham; $^{**}$P<0.01 vs. Model. SANFH, steroid-induced avascular necrosis of the femoral head; Sham, sham control group; Model, SANFH model group; Rb1, 200 mg/kg of Ginsenoside Rb1 group; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, chloramphenicol acetyltransferase; GSH-PX, glutathione peroxidase.

significantly upregulated in SANFH model rats, compared with the control group. Treatment with Ginsenoside Rb1 significantly reduced p65, TNF-α, IL-1β and IL-6 expression in SANFH rats compared with model rats.

*Ginsenoside Rb1 reduces oxidative stress in SANFH rats*. Next, it was demonstrated that MDA expression was increased (Fig. 4A), whereas SOD (Fig. 4B), CAT (Fig. 4C) and GSH-PX (Fig. 4D) expression was decreased in the model group, compared with the control. Consistently, treatment with Ginsenoside Rb1 significantly reduced MDA levels, and increased SOD, CAT and GSH-PX levels in SANFH rats, compared with the model group.

*Ginsenoside Rb1 reduces bone cell death in a rat model of SANFH*. The protective effects of Ginsenoside Rb1 against apoptosis in the femoral head were also explored. Specifically, Bax and p53 protein expression and caspase-3 activity were measured by western blotting (Fig. 5A). It was demonstrated that p53 (Fig. 5B), Bax (Fig. 5C) and caspase-3 (Fig. 5D) protein expression was significantly increased in SANFH rats, compared with the control group. Treatment with Ginsenoside Rb1 significantly reduced the induction of Bax, p53 and caspase-3 protein expression in SANFH rats.

*Ginsenoside Rb1 increases VEGF and VEGFR expression*. Western blotting was also used to determine whether Ginsenoside Rb1 exerted protective effects on VEGFR and VEGF expression (Fig. 6A). VEGF (Fig. 6B) and VEGFR (Fig. 6C) protein expression was significantly suppressed in the model group, compared with controls. Administration of

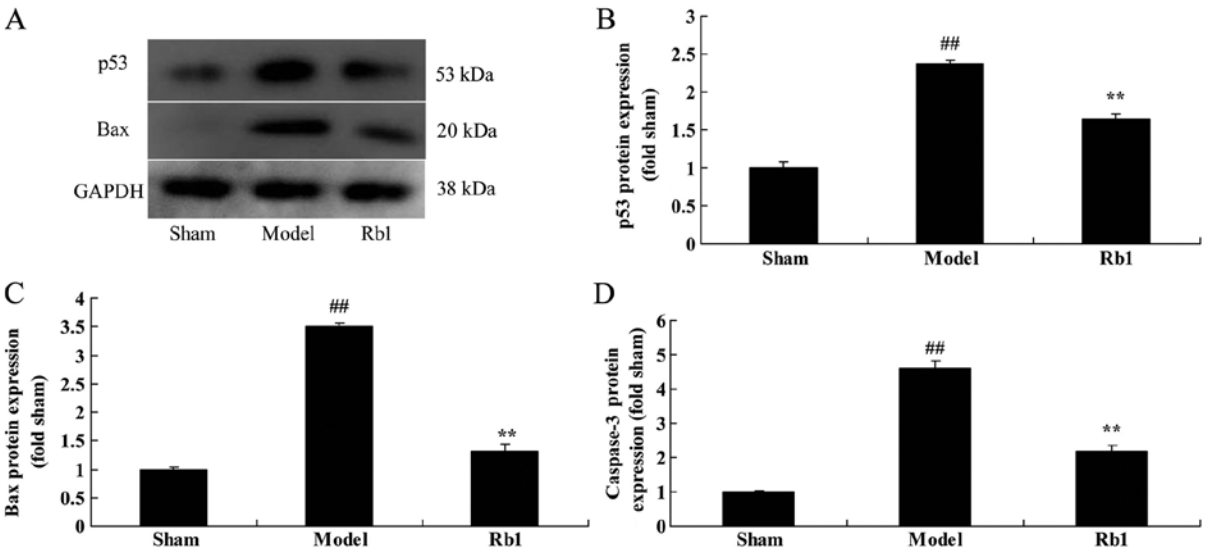


Figure 5. Protective effects of Ginsenoside Rb1 on bone cell apoptosis in a SANFH rat model. Protective effects of Ginsenoside Rb1 on Bax and p53 protein expression were determined by (A) western blotting assays and then statistical analysis was conducted to compare the (B) p53, (C) Bax and (D) caspase-3 protein expression levels in a SANFH rat model. Data are presented as the mean ± standard deviation. ##P<0.01 vs. Sham; **P<0.01 vs. Model. SANFH, steroid-induced avascular necrosis of the femoral head; Sham, sham control group; Model, SANFH model group; Rb1, 200 mg/kg of Ginsenoside Rb1 group.

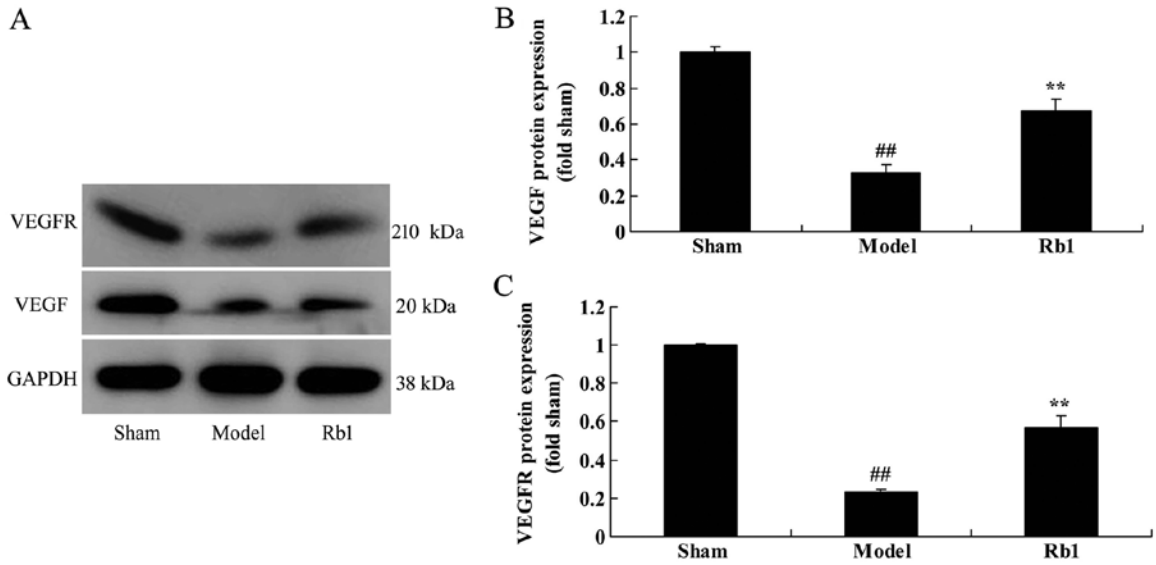


Figure 6. Protective effects of Ginsenoside Rb1 on VEGF in a SANFH rat model. Protective effects of Ginsenoside Rb1 on VEGFR and VEGF protein expression were determined by (A) western blotting assays and statistical analysis was conducted to evaluate (B) VEGFR and (C) VEGF protein expression in a SANFH rat model. Data are presented as the mean ± standard deviation. ##P<0.01 vs. Sham; **P<0.01 vs. Model. SANFH, steroid-induced avascular necrosis of the femoral head; Sham, sham control group; Model, SANFH model group; Rb1, 200 mg/kg of Ginsenoside Rb1 group; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

Ginsenoside Rb1 significantly induced VEGFR and VEGF protein expression in SANFH rats, compared with the untreated model group.

*Ginsenoside Rb1 on RUNX2 and BMP-2 protein expression in a rat model*. Finally, the protein expression of RUNX2 and BMP-2 was examined by western blot analysis (Fig. 7A). It was revealed that RUNX2 (Fig. 7B) and BMP-2 (Fig. 7C) protein expression in SANFH rats was lower than that of control group. Treatment with Ginsenoside Rb1 significantly induced RUNX2 and BMP-4 protein expression in SANFH rats, compared with SANFH model group.

## Discussion

Hyperlipidemia manifests in SANFH throughout the whole course of the disease (12). Reduced osteogenic differentiation slows the reparative process and accelerates femoral head necrosis (13). In addition, hormone administration results in hyperlipidemia, thrombosis and bone tissue ischemia (14). Together, this leads to the development of SANFH. In the present study, Ginsenoside Rb1 was demonstrated to be protective against steroid-induced avascular necrosis, which inhibited serum OST expression, reduced oxidative stress and inhibited bone cell apoptosis in a rat model of avascular necrosis. Similar

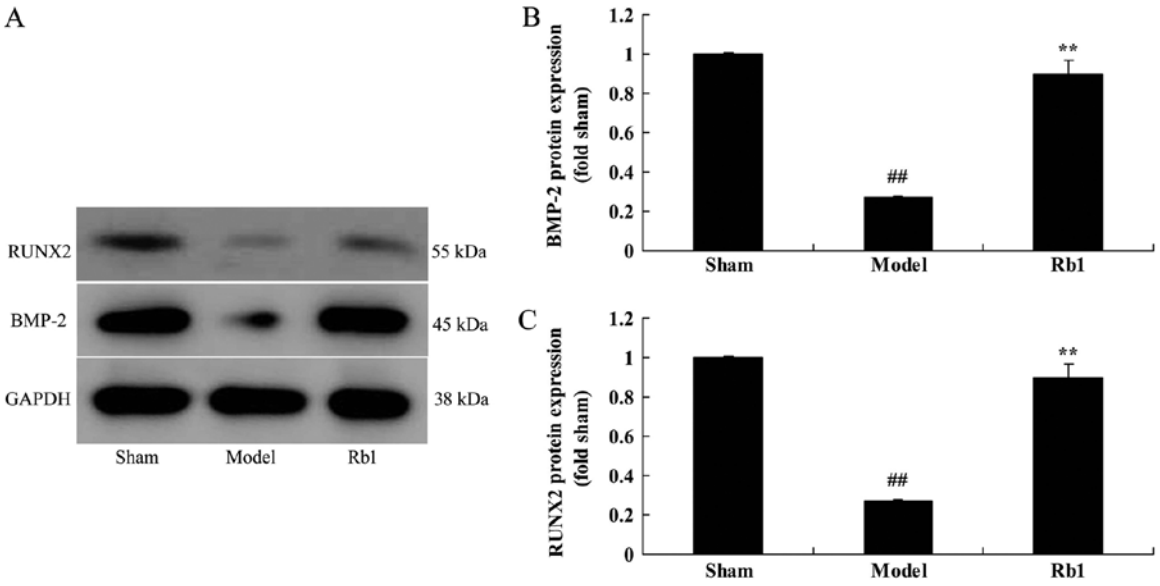


Figure 7. Protective effects of Ginsenoside Rb1 on RUNX2 and BMP-2 protein expression in a SANFH rat model. Protective effects of Ginsenoside Rb1 on RUNX2 and BMP-2 protein expression were determined by (A) western blotting assays and statistical analysis was conducted to evaluate (B) BMP-2 and (C) RUNX2 protein expression in a SANFH rat model. Data are presented as the mean ± standard deviation. $^{\#\#}$P<0.01 vs. Sham; $^{**}$P<0.01 vs. Model. SANFH, steroid-induced avascular necrosis of the femoral head; RUNX2, Runt related transcription factor 2; BMP, bone morphogenetic protein; Sham, sham control group; Model, SANFH model group; Rb1, 200 mg/kg of Ginsenoside Rb1 group.

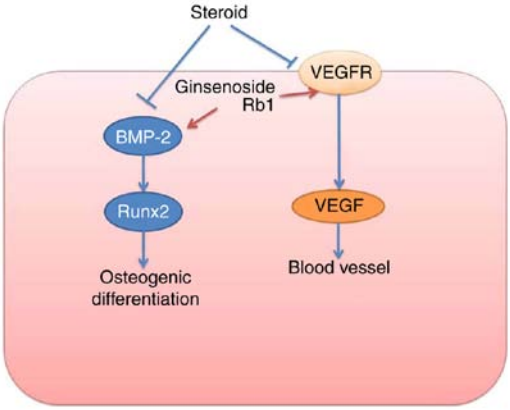


Figure 8. Effects of Ginsenoside Rb1 prevent the steroid-induced avascular necrosis of the femoral head through BMP-2 and VEGF signaling pathway. VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; RUNX2, Runt related transcription factor 2; BMP, bone morphogenetic protein.

to the results of the present study, Xiang *et al* (15) reported that Ginsenoside Rb1 inhibits osteoarthritis by downregulating Notch signaling, and Zhu *et al* (16) demonstrated that Ginsenoside Rb1 alleviates aluminum chloride-induced rat osteoblast dysfunction.

Organisms would induce a repair response following SANFH (17). The head of the femur begins to repair from the edge of the sequestrum and the surrounding living tissues (18). It is represented as revascularization, osteogenesis and absorption of dead bones. VEGF plays an important role in osteogenesis by promoting angiogenesis and inhibiting the apoptosis of chondrocytes and osteoblasts to affect osteogenesis by means of promoting bone turnover (19). These results indicate that Ginsenoside Rb1 significantly induces VEGFR and VEGF protein expression in SANFH rat. Lan *et al* (20) has reported that Ginsenoside Rb1 prevents homocysteine-induced dysfunction through via the VEGF/p38MAPK pathway.

BMP plays an essential role in bone growth and wound repair. Active mesenchymal cells are the target cells of BMP (21). Under the induction of BMP, muscular and perivascular mesenchymal cells can be differentiated into osteocytes, a process that is specific and irreversible (22). Recent studies have suggested that BMP-2 may first combine with and activate Type II and I receptors on the surface of the membrane (23). Secondly, intracellular signal transduction pathways of Smad may then be activated to induce the transcription of intranuclear target genes and protein expressions (21). Novel research has suggested that BMP-2 is the most representative in BMP family, with relatively high contents (23). Furthermore, osteogenic activity is relatively high and potent; separation and purification are relatively easy. Taken together, these findings confirm that Ginsenoside Rb1 significantly induced BMP2 protein expression in SANFH rats. Zhu *et al* (16) reported that Ginsenoside Rb1 alleviates aluminum chloride-induced rat osteoblast dysfunction through BMP-2 expression.

Runx2 is affected both by positive effects during osteoblast differentiation from marrow stroma cells and by negative modulation (24). The expression of Runx2 is upregulated after activation, thereby promoting the production of osteoblasts (25), which is quite significant for the early repair of SANFH. However, the activities of Runx2 are inhibited by peroxisome proliferator-activated receptor γ, whose high expression can inhibit the expression of Runx2 (26). Thus, the differentiation from bone marrow stromal cells to osteoblasts is reduced while differentiation to adipocytes is increased (27). This change will trigger worse femoral head necrosis. Taken together, the above findings illustrate that Ginsenoside Rb1 significantly induced RUNX2 protein expression in SANFH rats.

In conclusion, these outcomes indicate that Ginsenoside Rb1 exerts a positive protective effect on SANFH that is mediated by osteogenic differentiation targeted VEGFR/VEGF and RUNX2/BMP-4 signaling pathways (Fig. 8). Ginsenoside Rb1

may be a useful and novel protective drug for patients who require corticosteroid treatments and are at risk of developing SANFH.

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**Availability of data and materials**

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

**Authors' contributions**

TC designed the experiments. JY, DW and LP performed the experiments. JY and TC analyzed the data. TC wrote the manuscript. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

All procedures were approved by the Ethical Committee of Animal Experimentation at Southwest Medical University.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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