

Isopsoralen regulates PPAR- γ /WNT to inhibit oxidative stress in osteoporosis

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Received July 17, 2016; Accepted June 26, 2017

DOI: 10.3892/mmr.2017.7954

Abstract. The present study aimed to examine the effects of isopsoralen against postmenopausal osteoporosis in an ovariectomized rat model. The ovariectomized rats were treated with three days 10 mg/kg isopsoralen or with three days 20 mg/kg isopsoralen. Alkaline phosphatase, the oxidative stress indicators and caspase-3/9 were measured using ELISA assay kits. Reverse transcription-quantitative polymerase chain reaction was used to measure collagen type I (Col I), osteocalcin and osteoprotegerin mRNA levels. Wnt, β -catenin and peroxisome proliferators-activated receptor γ (PPAR- γ) were analyzed using western blot analysis. Isopsoralen suppressed mature adipocyte differentiation of C2C12 cells, inhibited serum calcium and urinary calcium levels, and reduced the structural scores of articular cartilage and cancellous bone in the proximal tibia metaphysis of mice with postmenopausal osteoporosis. Isopsoralen also promoted the activity of alkaline phosphatase and the mRNA expression levels of Col 1, osterix and osteopontin in mice with postmenopausal osteoporosis. Oxidative stress and activities of caspase-3/9 in the mice with postmenopausal osteoporosis were effectively suppressed by isopsoralen treatment, which upregulated the protein expression of Wnt/ β -catenin and downregulated the protein expression of PPAR- γ . These findings demonstrated that isopsoralen prevented osteoporosis through the regulation of PPAR- γ /WNT, inhibiting oxidative stress by targeting the PPAR- γ /WNT pathway. These results provide evidence of the potential targeted therapy for isopsoralen in the clinical treatment of postmenopausal osteoporosis.

Introduction

Osteoporosis is a systemic disease, which is characterized by low bone mineral density (BMD) and deterioration of skeletal microarchitecture, leading to increased risk of fragility fractures (1). The number of patients presenting clinically with osteoporosis is increasingly common; a major hazard of osteoporosis is the associated increased risk of fracture and the potential induction of numerous other complications (2). It has been shown in epidemiological investigations that osteoporosis has become a common disease in clinics, and the importance of its associated risks has been increasingly recognized by clinicians (2,3).

Osteoporosis is a type of metabolic disease, which is characterized by low BMD, deterioration of skeletal microarchitecture and increased risk of fragility fracture (4). It is associated with a diverse pathogenesis and complex molecular mechanisms, and has become an epidemic affecting patient quality of life. The human body lives in an oxygen-rich environment, and the metabolic process of oxygen produces reactive oxygen species (ROS) continuously. The body experiences oxidative stress when the balance between the generation of ROS and the elimination of ROS is disrupted as a result of aging and disease (5). Increasingly, it has been found that ROS-induced oxidative stress is important in osteoporosis; the excess of ROS regulates multiple signaling pathways through the activation or inhibition of multiple cytokines and enzyme activities, in addition to the upregulation or downregulation of the expression of receptor ligands. This affects the expression of endonuclear genes, accelerates the apoptosis of osteogenesis-related cells, including bone mesenchymal stem cells (BMSCs), osteoblasts and osteocytes, and increases the proliferation and differentiation of osteoclasts. This results in a reduced bone absorption rate relative to the bone formation rate, and alters the dynamic balance between osteoclasts absorbing bone tissue and osteoblasts forming bone tissue, resulting in osteoporosis (6,7).

As an essential cell transcriptional factor during the maturation process of adipocytes, the expression and activity of peroxisome proliferators-activated receptor γ (PPAR- γ) may determine the differentiation direction of mouse BMSCs into osteoblasts or adipocytes (8). In previous years, OS has attracted increased attention as a risk factor for osteoporosis, and investigations on the role of the PPAR- γ /WNT pathway in OS-mediated osteoporosis have been the most extensive and

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Key words: isopsoralen, osteoporosis, oxidative stress, peroxisome proliferators-activated receptor γ , WNT

thorough (9). Consequently, the focus on anti-osteoporosis has gradually changed from an estrogen-centered approach to an aging and oxidative stress-centered approach. In our previous study, isopsoralen inhibited the differentiation of mouse BMSCs into mature adipocytes in a concentration-dependent manner; in addition, the above-mentioned effects had marked effects on improving ovarian hormone deficiency-induced osteoporosis in addition to bone damage.

Traditional Chinese medicine has a history dating back several thousand years and, in traditional medicine use in China, the fruit of the *Psoralea corylifolia* has been used extensively for improving performance of the kidney, spleen and stomach. It is also termed *Fructus psoraleae*, Pogizhi and Hufeizi (10), and has been frequently used for treating fractures and osteoarthropathy, with marked effects (11). Clinically, *P. corylifolia* has also been used in treating dermatosis, cardiovascular lesions, tumors and asthma. Isopsoralen (Fig. 1), an isomer with psoralen, is the major effective component in the extract of *P. corylifolia* (12). Numerous investigations have revealed that psoralen is capable of promoting osteoblast differentiation and maturation, and stimulating bone formation (13). The present study aimed to determine how isopsoralen regulates osteoporosis and to elucidate the mechanism involved.

Materials and methods

Animals and experimental design. Six-week-old female Sprague-Dawley rats (weighing 140–160 g) were purchased from Inner Mongolia Medical University (Inner Mongolia, China) and housed in polycarbonate cages in temperature-controlled rooms ($22 \pm 2^\circ\text{C}$) with relative humidity of $55 \pm 5\%$ and a 12-h light/dark cycle. The experimental protocol was approved by the Animal Care and Use Review Committee of The Inner Mongolia People's Hospital. The rats were fed a standard laboratory diet and were provided with water *ad libitum* for an adaptation period of 7 days.

The rats were randomly distributed into four groups: Sham-operated control (sham), ovariectomized rats without treatment (osteoporosis), ovariectomized rats treated with isopsoralen 10 mg/kg/every 3 days (ISO-10), and ovariectomized rats treated with isopsoralen 20 mg/kg every 3 days (ISO-20). All groups were treated for 12 weeks. Following adaptation, the female ovariectomized rats were anesthetized with 2% isoflurane, and ovaries were removed bilaterally.

Calcium and urinary analysis. Blood was collected from the eye sockets of mice under 2% isoflurane, and serum and urine were collected following $4,000 \times g$ centrifugation for 10 min at 4°C . The serum and urinary calcium levels were determined using a Technicon SMAC analyzer (Technicon Instruments Corporation, Tarrytown, NY, USA). Serum levels of leptin and parathyroid hormone (PTH) were determined using Novartis Pharma Ag with a Luminex 200™ Multiplexing instrument. A high-resolution desktop micro-CT system (SkyScan 1174v2; Bruker MicroCT, Kontich, Belgium) was used to analyze the bone mineral density (BMD).

ELISA analysis. Blood was collected from the eye sockets under 2% isoflurane and serum was collected following $4,000 \times g$ centrifugation for 10 min at 4°C . The levels of

alkaline phosphatase (ALP), the oxidative stress indicators, superoxide dismutase (SOD), malondialdehyde (MDA), glutathione (GSH) and glutathione peroxidase (GSH-PX), and the activities of caspase-3/9 were measured using ELISA assay kits (Elabscience Biotechnology Co., Ltd., Wuhan, China) according to the manufacturer's protocol, respectively.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was prepared from cartilage tissue using an RNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA). cDNA was synthesized using 1–2 μg of total RNA using reverse transcriptase (Takara Bio, Inc., Otsu, Japan). The ABI 7500 sequencing detection system (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used for qPCR analysis using the SYBR Premix Ex Tag kit (Takara Bio, Inc.) with 200 ng cDNA (total reaction volume: 10 μl ; 1–2 μl cDNA, 2 μl primers, 2 μl SYBR and 4 μl water). The primer sets used are listed in Table I. The detector was programmed with the following PCR conditions: 40 cycles of 15 sec denaturation at 95°C , 40 sec amplification at 60°C and 30 sec at 72°C . The mRNA levels were calculated using the $2^{-\Delta\Delta\text{C}_q}$ analysis method (14).

Western blot analysis. Cartilage tissues were collected and lysed in lysis buffer. The protein content of the supernatant was determined using a BCA™ protein assay kit (Beyotime Institute of Biotechnology, Haimen, China) following $10,000 \times g$ centrifugation for 10 min at 4°C . Total protein (50 μg) was separated by 8–10% SDS-PAGE and subsequently electrotransferred onto a PVDF membrane (EMD Millipore, Bedford, MA, USA). The membrane was blocked with 5% non-fat milk for 1 h at 37°C and incubated with the indicated antibodies: Anti-Wnt (cat. no. sc-5630; 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) anti- β -catenin (cat. no. sc-7199; 1:500; Santa Cruz Biotechnology, Inc.), ant-PPAR- γ (cat. no. sc-7196; 1:500; Santa Cruz Biotechnology, Inc.) and anti-GAPDH (cat. no. sc-25778; 1:500; Santa Cruz Biotechnology, Inc.) at 4°C overnight. Following washing three times for 15 min with TBST, the membrane was detected by incubation with anti-rabbit horse-radish peroxidase (HRP)-conjugated secondary antibodies (cat. no. sc-2004; 1:2,000; Santa Cruz Biotechnology, Inc.) for 1 h, and immunodetection with enhanced chemiluminescence (Thermo Fisher Scientific, Inc.) and quantified by Image Lab version 3.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Data are expressed as the mean \pm standard error of the mean using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). The experimental groups were compared using one-way analysis of variance and Duncan's test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Isopsoralen regulates urine calcium/creatinine (Ca/Cr) in osteoporosis. At 12 weeks post-isopsoralen treatment, the urine level of Ca/Cr in the osteoporosis model group was higher, compared with that in the sham group (Fig. 2). Treatment with 10 or 20 mg/kg isopsoralen effectively inhibited the increased urine Ca/Cr in the rats with osteoporosis, compared with the osteoporosis rat model group (Fig. 2).

Table I. Primer sets used for reverse transcription-quantitative polymerase chain reaction analysis.

Gene	Primer (5'-3')
Col I	F: ACCTAAGGGTACCGCTGGA R: TCCAGCTTCTCCATCTTTGC
OCN	F: TCTCTCTGACCTCACAGATGCC R: TCCAGCTTCTCCATCTTTGC
OPG	F: TGTTCTTACCAAGATTATACCAAAT R: CGCTCGATTTGCAGGTCTTT
GAPDH	F: ACCACAGTCCATGCCATCAC R: TCCACCACCCTGTTGCTGTA

Col I, collagen type I; OCN, osteocalcin; OPG, osteoprotegerin; F, forward; R, reverse.

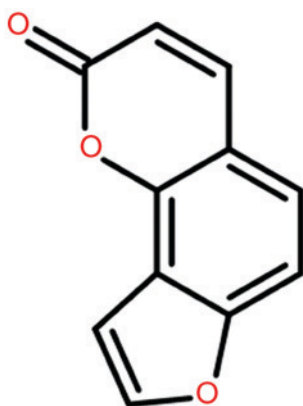


Figure 1. Chemical structure of isopsoralen.

Isopsoralen regulates BMD and structure of the proximal tibial metaphysis (PTM) in osteoporosis. There was a significant decrease in BMD and increase in PTM scores in the osteoporosis model rats, compared with the levels in the sham group (Fig. 3A and B). Treatment with 10 or 20 mg/kg isopsoralen significantly reversed the inhibited BMD and increased PTM scores in the rats with osteoporosis, compared with those in the osteoporosis rat model group (Fig. 3A and B).

Isopsoralen regulates leptin and calcium in osteoporosis. Inhibition of leptin levels and increasing levels of calcium were observed in the osteoporosis model, compared with levels in the sham group (Fig. 4A and B). However, treatment with 10 or 20 mg/kg isopsoralen significantly increased the levels of leptin and reduced calcium in the rats with osteoporosis, compared with the osteoporosis rat model group (Fig. 4A and B).

Isopsoralen regulates levels of ALP, collagen type I (Col I), osteocalcin (OCN) and osteoprotegerin (OPN) in osteoporosis. The present study investigated the pro-osteogenic effects of isopsoralen on ALP, Col I, OCN and OPN in the rats with osteoporosis. As shown in Fig. 5A-D, the levels of ALP, Col I, OCN and OPN were stimulated in the osteoporosis

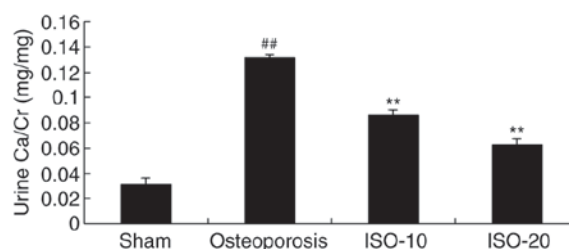


Figure 2. Isopsoralen regulates urine Ca/Cr in osteoporosis. ##P<0.01, compared with the sham group; **P<0.01, compared with the osteoporosis model group. ISO, isopsoralen; Ca/Cr, calcium/creatinine; Sham, sham group; Osteoporosis, osteoporosis model group; ISO-10, 10 mg/kg ISO group.

model rat, compared with the levels in the sham group. In the later stage, the induced activities of ALP, Col I, OCN and OPN were significantly inhibited by 10 and 20 mg/kg isopsoralen in the rats with osteoporosis (Fig. 5A-D).

Isopsoralen regulates oxidative stress in osteoporosis. To detect the anti-oxidative effects of isopsoralen on oxidative stress in the osteoporosis rat model, the activities of SOD, MDA, GSH and GSH-PX were examined. In the osteoporosis model rats, it was found that the activities of SOD, GSH and GSH-PX were decreased, and the activity of MDA was increased, compared with activities in the sham group (Fig. 6A-D). Treatment with 10 or 20 mg/kg isopsoralen significantly decreased the activity of MDA, and increased the activities of SOD, GSH and GSH-PX in the rats with osteoporosis (Fig. 6A-D).

Isopsoralen regulates caspase-3/9 activity in osteoporosis. In order to examine the effect of isopsoralen on the apoptosis in bone of rats with osteoporosis, the present study examined caspase-3/9 activity in the osteoporosis rat model. As shown in Fig. 7A and B, significant increases in caspase-3/9 activity were observed in the osteoporosis rat model, compared with the sham group. Treatment with 10 or 20 mg/kg isopsoralen significantly weakened caspase-3/9 activity in the rats with osteoporosis (Fig. 7A and B).

Isopsoralen regulates the protein expression of WNT in osteoporosis. To confirm the osteogenic effects of isopsoralen on osteoporosis, the protein expression of WNT was measured using western blot analysis. There was a significant decrease in the protein expression of WNT in the osteoporosis model rat, compared with that in the sham group (Fig. 8A and B). Treatment with 10 or 20 mg/kg isopsoralen significantly induced the protein expression of WNT in the rats with osteoporosis (Fig. 8A and B).

Isopsoralen regulates the protein expression of β -catenin in osteoporosis. The present study also examined whether the protein expression of β -catenin is involved in the osteogenic effects of isopsoralen on osteoporosis. It was found that the protein expression of β -catenin was significantly inhibited in the osteoporosis rat model, compared with that in the sham group (Fig. 9A and B). However, the inhibition of the protein expression of β -catenin was significantly reduced in the rats with osteoporosis by 10 or 20 mg/kg isopsoralen (Fig. 9A and B).

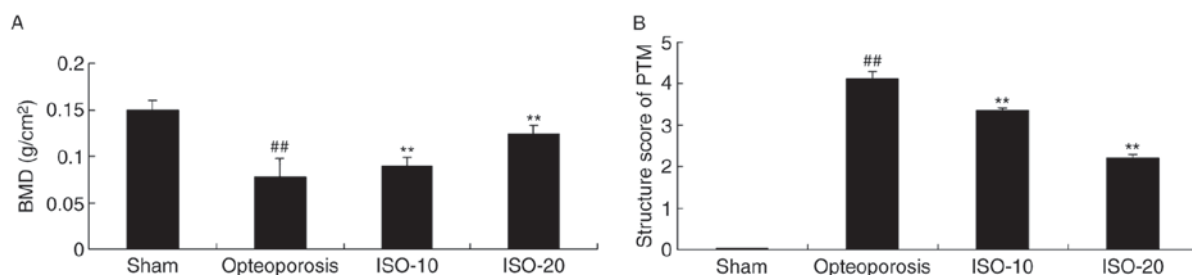


Figure 3. Isopsoralen regulates BMD and PTM in osteoporosis. Isopsoralen regulated (A) BMD and (B) PTM in osteoporosis. ## $P < 0.01$, compared with the sham group; ** $P < 0.01$, compared with the osteoporosis model group. ISO, isopsoralen; Sham, sham group; Osteoporosis, osteoporosis model; ISO-10, 10 mg/kg ISO; ISO-20, 20 mg/kg ISO. BMD, bone mineral density; PTM, proximal tibia metaphysis.

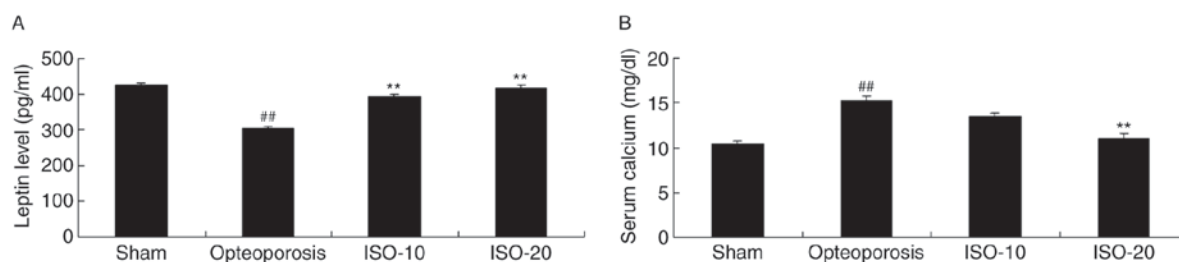


Figure 4. Isopsoralen regulates leptin and calcium in osteoporosis. Isopsoralen regulated (A) leptin and (B) calcium in osteoporosis. ## $P < 0.01$, compared with the sham group; ** $P < 0.01$, compared with the osteoporosis model group. ISO, isopsoralen; Sham, sham group; Osteoporosis, osteoporosis model; ISO-10, 10 mg/kg ISO; ISO-20, 20 mg/kg ISO.

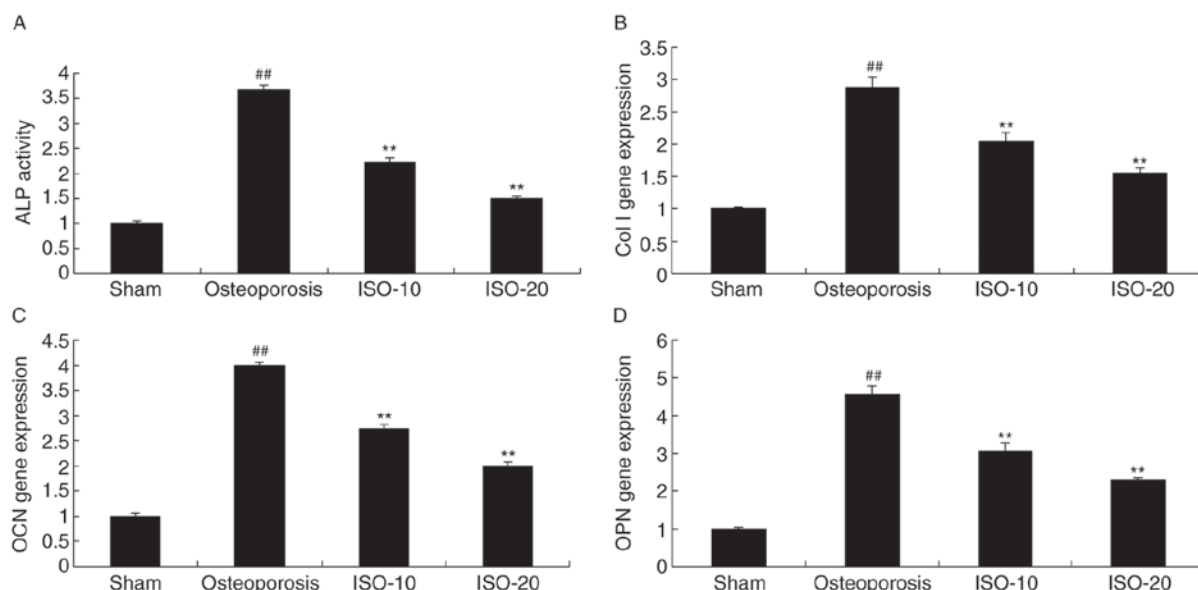


Figure 5. Isopsoralen regulates ALP, Col I, OCN and OPN in osteoporosis. Isopsoralen regulated (A) ALP, (B) Col I, (C) OCN and (D) OPN in osteoporosis. ## $P < 0.01$, compared with the sham group; ** $P < 0.01$, compared with the osteoporosis model group. ISO, isopsoralen; Sham, sham group; Osteoporosis, osteoporosis model; ISO-10, 10 mg/kg ISO; ISO-20, 20 mg/kg ISO; ALP, alkaline phosphatase; Col I, collagen type I; OCN, osteocalcin; OPN, osteoprotegerin.

Isopsoralen regulates the protein expression of PPAR- γ in osteoporosis. Finally, the present study investigated whether PPAR- γ affects the osteogenic effects of isopsoralen on osteoporosis. As shown in Fig. 10A and B, the protein expression of PPAR- γ was significantly induced in the osteoporosis model rats, compared with that in the sham group. Treatment with 10 or 20 mg/kg isopsoralen significantly suppressed the protein expression of PPAR- γ in the rats with osteoporosis (Fig. 10A and B).

Discussion

Numerous studies have been performed on psoralen, the results of which demonstrate that psoralen has excellent protective effect on the steady state of bone health (15). Osteoporosis is a systemic disease, which is characterized by low BMD and the deterioration of skeletal microarchitecture, leading to increased risk of fragility fractures (16). Osteoporosis can be divided into the two broad categories

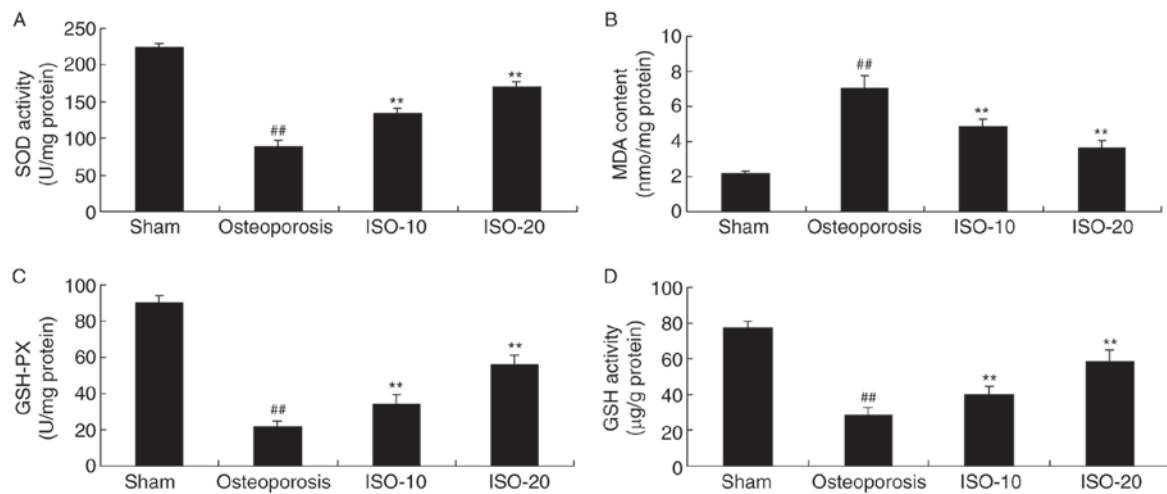


Figure 6. Isopsoralen regulates oxidative stress in osteoporosis. Isopsoralen regulated (A) SOD, (B) MDA, (C) GSH and (D) GSH-PX in osteoporosis. ^{##}P<0.01, compared with the sham group; ^{**}P<0.01, compared with the osteoporosis model group. ISO, isopsoralen; Sham, sham group; Osteoporosis, osteoporosis model; ISO-10, 10 mg/kg ISO; ISO-20, 20 mg/kg ISO; SOD, superoxide dismutase; MDA, malondialdehyde; GSH, glutathione; GSH-PX, glutathione peroxidase.

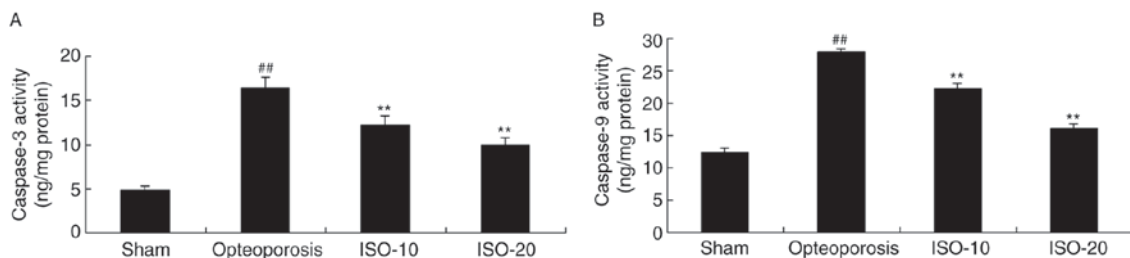


Figure 7. Isopsoralen regulates caspase-3/9 activity in osteoporosis. Isopsoralen regulated the activity of (A) caspase-3 and (B) caspase-9 in osteoporosis. ^{##}P<0.01, compared with the sham group; ^{**}P<0.01, compared with the osteoporosis model group. ISO, isopsoralen; Sham, sham group; Osteoporosis, osteoporosis model; ISO-10, 10 mg/kg ISO; ISO-20, 20 mg/kg ISO.

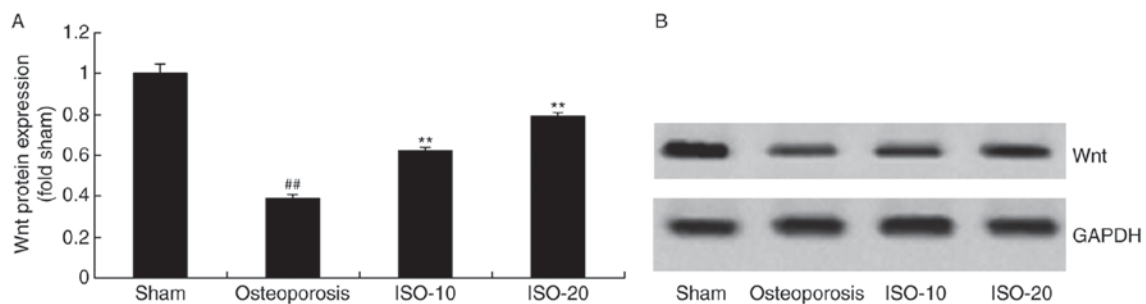


Figure 8. Isopsoralen regulates the protein expression of WNT in osteoporosis. Isopsoralen regulated the protein expression of WNT in osteoporosis, determined using (A) statistical analysis and (B) western blot analysis. ^{##}P<0.01, compared with the sham group; ^{**}P<0.01, compared with the osteoporosis model group. ISO, isopsoralen; Sham, sham group; Osteoporosis, osteoporosis model; ISO-10, 10 mg/kg ISO; ISO-20, 20 mg/kg ISO.

of primary and secondary osteoporosis; the former can be further divided into post-menopausal osteoporosis (type I), senile osteoporosis (type II) and idiopathic osteoporosis; and the latter refers to any disease affecting the bone physiology or drugs leading to osteoporosis, for example, osteoporosis induced by long-term administration of a high-dose glucocorticoid (2,17). Generally, post-menopausal osteoporosis occurs within 5-10 years of menopause in women, whereas senile osteoporosis occurs in women >70 years old, and idiopathic osteoporosis primarily occurs in adolescents, with an unclear pathogenesis (18). The present study is the first, to

the best of our knowledge, to demonstrate that isopsoralen treated or prevented osteoporosis, leading to decreased urine Ca/Cr, decreased BMD and PTM, and increased leptin and decreased calcium in rats with osteoporosis. Ming *et al* (19) also reported that isopsoralen prevented anti-osteoporotic and BMSC differentiation in rats.

OCN and Runt-related transcription factor 2 (RUNX2) are key regulatory factors in osteoblast differentiation, which is important during bone formation. They are also used in investigations to measure the osteogenic and adipogenic abilities of cells. In the present study, it was found that isopsoralen

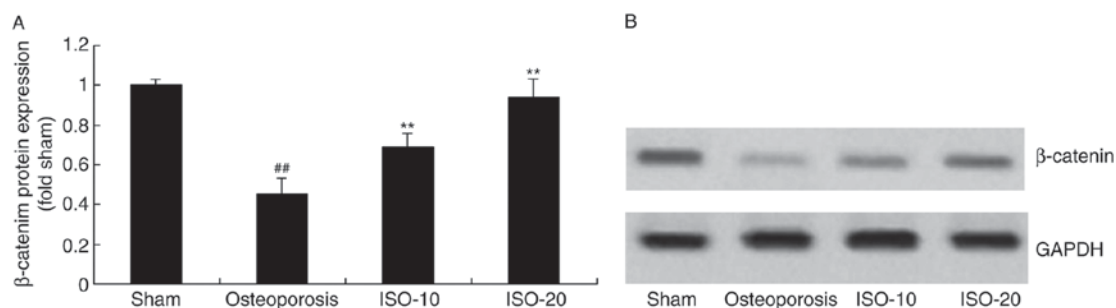


Figure 9. Isopsoralen regulates the protein expression of β -catenin in osteoporosis. Isopsoralen regulated the protein expression of β -catenin in osteoporosis, determined using (A) statistical analysis and (B) western blot analysis. ^{##} $P < 0.01$, compared with the sham group; ^{**} $P < 0.01$, compared with the osteoporosis model group. ISO, isopsoralen; Sham, sham group; Osteoporosis, osteoporosis model; ISO-10, 10 mg/kg ISO; ISO-20, 20 mg/kg ISO.

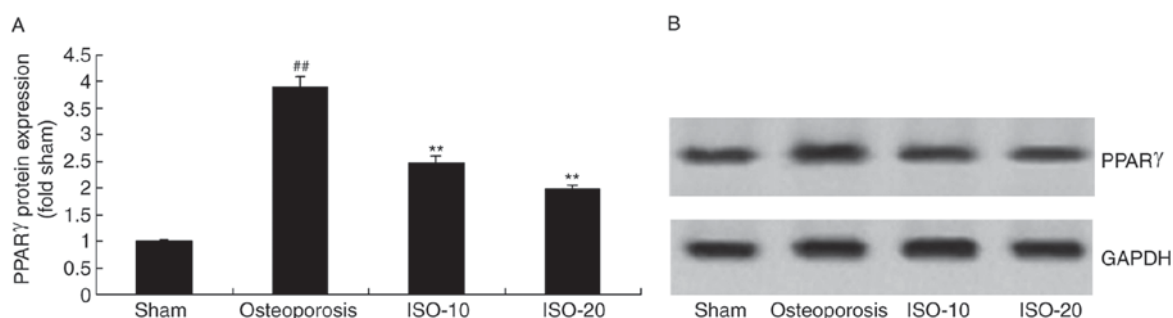


Figure 10. Isopsoralen regulates the protein expression of PPAR- γ in osteoporosis. Isopsoralen regulated the protein expression of PPAR- γ in osteoporosis, determined using (A) statistical analysis and (B) western blot analysis in osteoporosis. ^{##} $P < 0.01$, compared with the sham group; ^{**} $P < 0.01$, compared with the osteoporosis model group. ISO, isopsoralen; Sham, sham group; Osteoporosis, osteoporosis model; ISO-10, 10 mg/kg ISO; ISO-20, 20 mg/kg ISO; PPAR- γ , peroxisome proliferators-activated receptor γ .

significantly inhibited the activities of ALP, Col I, OCN and OPN in rats with osteoporosis.

Bone cells are a type of long-life cell, which is more susceptible to oxidative stress than osteoblasts and osteoclasts (20). Several experiments on rodents and humans have indicated that under oxidative stress induced by aging of the body, the number of bone cells gradually decreases, with weakened viability (21). The extracellular matrix in tissue is comprised of organic and inorganic components; the organic components are predominantly composed of a variety of collagens, whereas the inorganic components are hydroxyapatite crystals (5). The abnormally increased levels of ROS, in addition to the matrix metalloproteinase and cysteine protease within osteoclasts, may damage the sulfhydryl group and amino group of the protein, leading to protein denaturation and crosslinking, and damage to collagen and fibronectin (22). In the present study, isopsoralen significantly decreased the activity of MDA, and increased the activities of SOD, GSH and GSH-PX in the rats with osteoporosis. Feng *et al* (23) reported that isopsoralen also effectively inhibited H_2O_2 -induced oxidative damage in HLE-B3 cells.

In bone metabolic pathways, the signal transduction of multiple pathways are involved in cell differentiation, among which the WNT signaling pathway has been a consistent focus of investigations. WNT can control the fate of several types of cells, is involved in the processes of cell proliferation, differentiation, migration, polarization and apoptosis, and sustains cellular dynamic balance (24). The WNT pathway is the most extensively investigated of the WNT signaling

pathways. When the WNT signaling pathway is activated, the extracellular WNT protein binds with the cell surface specific receptor, transmembrane receptor frizzled protein, and the co-receptor, low-density lipoprotein receptor associated protein 5/6 (LRP5/6) to form the WNT-Fzd-LRP5/6 complex. This complex activates the disheveled protein (Dvl) in T cells, and the activated Dvl sends signals to the cytoplasm to promote the binding of Dvl with the Fzd receptor, leading to the binding of LRP5/6 with the axis protein complex and phosphorylation. In this manner, the process of the downstream casein kinase-adenomatous polyposis protein-glycogen synthase kinase 3P-axis protein complex in the degradation and acidification of catenin in the cytoplasm is inhibited. As a result, β -catenin accumulates in the cell, enters the cell nucleus, and forms a complex with T cytokines and lymphoid enhancement factor; this specifically binds with the whip gene transcription promoter and jointly regulates the transcription process, leading to WNT signal activation (25-27). The classical WNT signaling pathway inhibits osteoclast formation through driving the differentiation of BMSCs into osteoblasts, resulting in the promotion of bone formation and affecting bone remodeling (9). The present study revealed that isopsoralen significantly weakened caspase-3/9 activity and induced the protein expression of WNT/ β -catenin in rats with osteoporosis.

As described above, OCN and RUNX2 are key regulatory factors in osteoblast differentiation, which are important during bone formation, and examined in studies for measuring the osteogenic and adipogenic abilities of cells (28). As

osteoblasts and adipocytes are derived from BMSCs, the results of the present study suggested that the mechanism of action of isoporsalen against osteoporosis may be to exert its estrogen-like effect, and regulate the balance between RUNX2 and PPAR- γ , thus inhibiting the differentiation of BMSCs into adipocytes (8). In the present study, isoporsalen significantly suppressed the protein expression of PPAR- γ in the rats with osteoporosis.

In conclusion, the findings of the present study showed that isoporsalen effectively inhibited urine Ca/Cr, restored the inhibition of BMD and increase of PTM, and increased leptin and decreased calcium in rats with osteoporosis. This may occur through the regulation of PPAR- γ /WNT to inhibit oxidative stress in osteoporosis. Therefore, isoporsalen is a promising candidate for development as a therapeutic agent against osteoporosis in postmenopausal women.

Acknowledgements

This study was supported by the Natural Science Foundation of China (grant no. 81560368).

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