

# Celastrol inhibits glucocorticoid-induced osteoporosis in rat via the PI3K/AKT and Wnt signaling pathways

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**Abstract.** Modern pharmacological studies revealed that Celastrol exhibits anti-inflammation, anti-bacteria, anti-virus, anti-fertility, insect-resistance functions and has been used for the treatment of rheumatism, rheumatoid arthritis, blood diseases, skin diseases and agricultural insecticide. The present study aimed to investigate the effects of Celastrol on glucocorticoid-induced osteoporosis (GIOP) and the underlying molecular mechanisms. The findings of the current study revealed that Celastrol reduced body weight, urine calcium/creatinine, tartrate-resistant acid phosphatase 5b, C-terminal telopeptide of type I collagen, and induced osteocalcin in GIOP rats. In addition, alkaline phosphatase, triiodothyronine receptor auxiliary protein and cathepsin K mRNA expression levels were effectively suppressed, and osteocalcin, bone morphogenetic protein 2, type I collagen and runt-related transcription factor 2 mRNA expression levels were effectively induced in osteoporosis rats treated with Celastrol. Celastrol inhibited prostaglandin E2 and caspase-3 protein expression levels, and induced phosphoinositide 3-kinase (PI3K), phosphorylated-protein kinase B (AKT) and glycogen synthase kinase-3 phosphorylation, Wnt and  $\beta$ -catenin protein expression in GIOP rats. The present study demonstrated that Celastrol may inhibit GIOP in rats via the PI3K/AKT and Wnt signaling pathways.

## Introduction

Osteoporosis is a systemic bone disease characterized by low bone mass, damaged bone microstructure, increased fragility and susceptibility to fractures (1). Osteoporosis may be divided into primary osteoporosis and secondary osteoporosis.

The former is subdivided into postmenopausal, elderly and idiopathic osteoporosis, and the latter includes osteoporosis caused by any disease or drug that affects the physiological function of bones, such as long-term and high-dose intake of glucocorticoids (2). Generally speaking, postmenopausal osteoporosis occurs in women during menopause, elderly osteoporosis affects people aged >70, and idiopathic osteoporosis mainly occurs in teenagers, although its pathogenesis remains to be elucidated (3). It has been previously reported that there are tens of millions of female and male osteoporosis patients in the United States, and billions of Chinese people suffer from low bone mass (3). Minor trauma may lead to fractures, teratogenesis, disability or death and other serious adverse consequences in osteoporosis patients, and therefore, osteoporosis has become one of primary factors affecting the quality of life of the elderly (4).

Under the action of the Wnt signaling pathway, mesenchymal stem cells differentiate into osteoblasts (5). The classic Wnt/ $\beta$ -catenin signal pathway in osteoblasts also regulates the formation of osteoclasts (6). Osteoblasts promote the expression of two factors required in the formation of osteoclasts, macrophage colony-stimulating factor and receptor activator for nuclear factor- $\kappa$ B ligand (RANKL). Osteoblasts also secrete and express osteoprotegerin (OPG), which is the decoy receptor of RANKL, and binding to osteoprotegerin may inhibit the interaction of RANK/RANKL, to inhibit the formation of osteoclasts (7). In osteoclast progenitor cells, when RANKL activates its receptor, osteoclasts will be stimulated to produce reactive oxygen species (7,8). Therefore, RANKL and OPG are key molecules bridging bone formation and bone resorption in bone remodeling.

Phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) is one of the most important signaling pathways that regulate cell proliferation, differentiation, survival, migration and metabolism (9). A previous study demonstrated that many signaling molecules involved in ossification selectively activate genes associated with the PI3K/AKT signaling pathway, and disturb the dynamic balance between bone formation and bone resorption during bone remodeling via the regulation of osteoblast and osteoclasts; therefore, this signaling pathway has a very important role in the incidence and development of osteoporosis (10).

*Celastrus orbiculatus* Thunb. belongs to the *Celastrus* genus of the Celastraceae family, and its root, stem, fruit and

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leaves may be used as medicine. It has been demonstrated that Celastrol (Fig. 1) has anti-oxidative and anti-inflammatory properties, inhibits atherosclerosis by lipoprotein oxidative modification and prevents against inflammation (11). Previous studies have revealed that Celastrol has anti-inflammatory, anti-bacterial, anti-viral, anti-fertility and insect-resistance functions, and has been used for the treatment of rheumatism, rheumatoid arthritis, blood diseases, skin diseases and as an agricultural insecticide (11,12). In the present study, bioinformatics analysis was used to investigate the effects of Celastrol on glucocorticoid-induced osteoporosis (GIOP) and the potential underlying molecular mechanisms.

## Materials and methods

**Animal treatment.** Male C57BL/6J mice (8-weeks old, 20-22 g, n=30) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). All mice were provided with food and water *ad libitum*, and were housed at a temperature of 22-23°C, a humidity of 55-60% and a 12/12 h light/dark cycle. The mice were randomly divided into three groups: i) Vehicle group (n=10); ii) GIOP model group (n=10); and iii) Celastrol treatment group (n=10). Mice were injected intramuscularly with 5 mg/kg body weight dexamethasone three times a week for 12 weeks. Mice in the Celastrol treatment group were injected with a daily dose of 1 mg/kg Celastrol (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 12 weeks. The study was approved by the Ethics Committee of the Department of Minimally Invasive Spine Surgery, The 309th Hospital of the People's Liberation Army (Beijing, China). Following treatment with Celastrol, body weight was determined. Urine calcium (cat. no. C004-2), creatinine (cat. no. A032) and tartrate resistant acid phosphatase-5b (TRACP-5b; cat. no. A058) were quantified using ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted using TRIzol reagent (Qiagen, Inc., Valencia, CA, USA). cDNA was synthesized using a High-Capacity cDNA Reverse Transcription kit (cat. no. 4368813; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). A RT-qPCR instrument (model ABI 7300) was used to analyze alcurin-like protease (ALP), triiodothyronine receptor auxiliary protein (TRAP), cathepsin K, osteocalcin, bone morphogenetic protein 2 (BMP-2), type I collagen, runt-related transcription factor 2 (Runx-2) mRNA expression levels using a SYBR Green-containing PCR kit (Shanghai GenePharma, Co., Ltd., Shanghai, China). The primer sequences used for qPCR were as follows: ALP forward, 5'-CCAGGGCGTACGGAGGCC ATT-3' and reverse, 5'-GACCAAATTACGGCGTAGCCT C-3'; TRAP forward, 5'-AGCATAAGGGTCCAAGTCCAA-3' and reverse, 5'-TACCAA AAGCGGCTAGT TA-3'; cathepsin K forward, 5'-AGGCGGAGGTCGATGCCCCG-3' and reverse, 5'-CACGATGATGTACCCCTCGATGT-3'; osteocalcin forward, 5'-ATGAGAGCCCTCACTCTCT-3' and reverse, 5'-CTTGGACACAAAGGCTGCAC-3'; BMP-2 forward, 5'-CAGCTTCCACCATGAAGAAT-3', and reverse, 5'-CCAACCTGGTGTCCAAAAGT-3'; type I collagen forward, 5'-CCTGGATGCCATCAAAGTCT-3', and reverse,

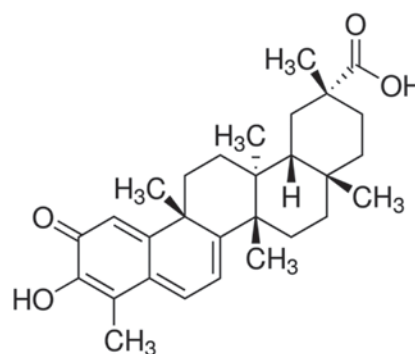


Figure 1. Chemical structure of Celastrol.

5'-ACTGCAACTGGAATCCATCG-3'; Runx-2 forward, 5'-CTCCCTGAAGTCTGCACCAA-3', and reverse, 5'-GTT CTGAAGCACCTGAAATGCG-3'; and GAPDH forward, 5'-ACAGGGGAGGTGATAGCATT-3' and reverse, 5'-GAC CAAAAGCCTTCATACATCTC-3'. The PCR conditions were as follows: Initial denaturation for 10 min at 95°C; followed by 40 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 60°C and a final extension for 30 sec at 72°C. The method for quantification used was the  $2^{-\Delta\Delta C_q}$  method (13).

**Western blot analysis.** Total proteins were extracted from tissue samples using radioimmunoprecipitation assay (Thermo Fisher Scientific, Inc.) and the protein concentration was quantified in triplicate using the Pierce™ bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). A total of 25 µg was subjected to 8-12% SDS-PAGE and directly transferred to nitrocellulose membranes (Sigma-Aldrich; Merck KGaA). Membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h at 37°C and then hybridized with the following primary antibodies: Anti-Wnt (cat. no. ab32249; 1:500; Abcam, Cambridge, UK), anti-β-catenin (cat. no. ab16051; 1:500; Abcam), PI3K (cat. no. sc-7174; 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), phosphorylated (p)-AKT (cat. no. sc-7985-R; 1:500; Santa Cruz Biotechnology, Inc.), p-glycogen synthase kinase-3 (GSK-3; cat. no. sc-81497; 1:500; Santa Cruz Biotechnology, Inc.), prostaglandin E2 (PGE-2; cat. no. ab96189; 1:500; Abcam), caspase-3 (cat. no. sc-98785; 1:500; Santa Cruz Biotechnology, Inc.) and GAPDH (cat. no. sc-25778; 1:5,000; Santa Cruz Biotechnology, Inc.) at 4°C overnight. The membranes were incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibodies (cat. no. 7074; 1:5,000; Cell Signaling Technology, Inc., Danvers, MA, USA) at the room temperature in the dark for 2 h. The blots were developed using enhanced chemiluminescence plus kits (GE Healthcare, Chicago, IL, USA), and densitometric analysis was performed using Image\_Lab\_3.0 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Statistical analysis.** All experimental data are presented as the mean ± standard error of the mean. Comparison between groups was performed using a one-way analysis of variance followed by Tukey's Honest Significant Difference post-hoc test.  $P < 0.05$  was considered to indicate a statistically significant difference.

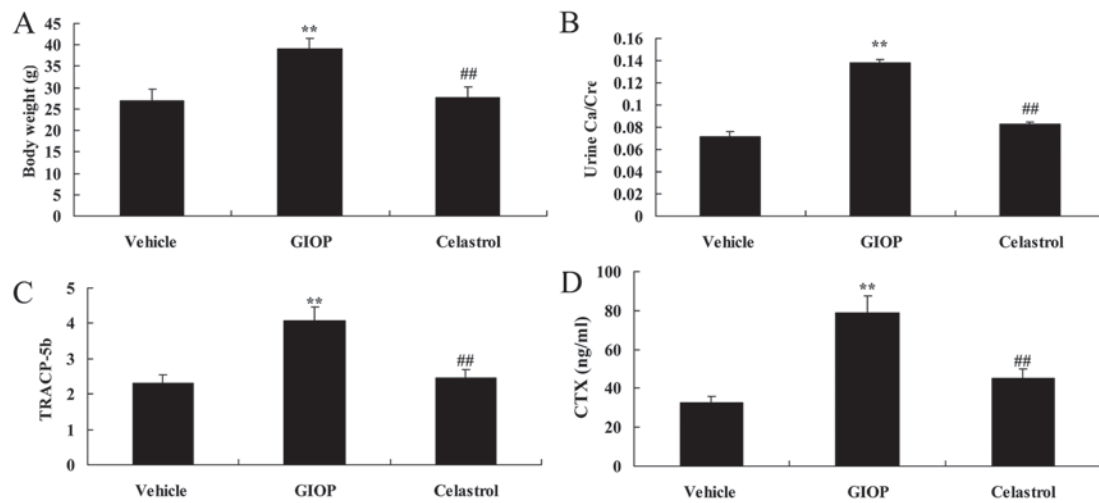


Figure 2. Celastrol inhibits body weight, urine Ca/Cre, TRACP-5b and CTX. Celastrol inhibited (A) body weight, (B) urine Ca/Cre, (C) TRACP-5b and (D) CTX. \*\*P<0.01 vs. vehicle group, ##P<0.01 vs. GIOP model group. CTX, C-terminal telopeptide of type I collagen; GIOP, glucocorticoid-induced osteoporosis model group; Ca/Cre, calcium/creatinine; TRACP-5b, tartrate-resistant acid phosphatase-5b.

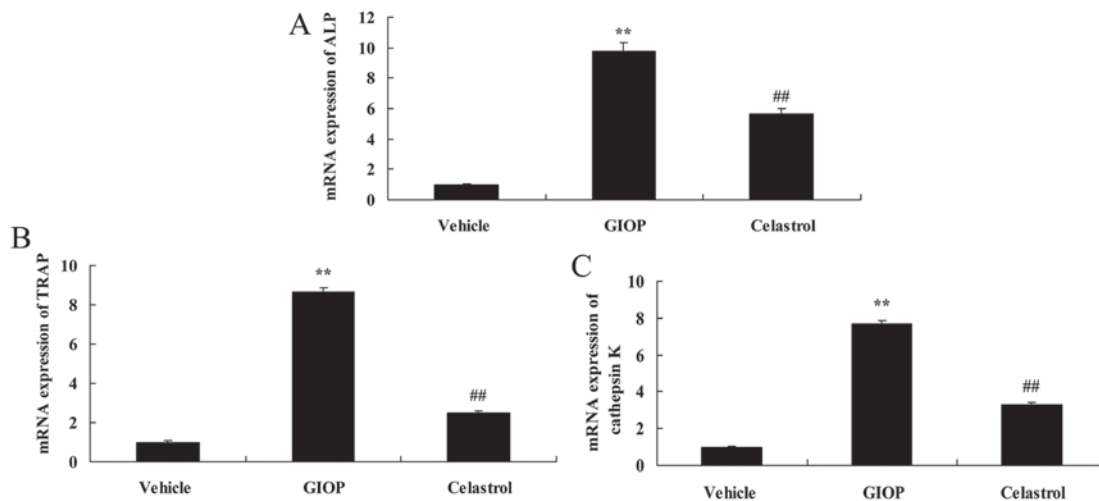


Figure 3. Celastrol inhibits ALP, TRAP and cathepsin K mRNA expression levels. Celastrol inhibited (A) ALP, (B) TRAP and (C) cathepsin K mRNA expression. \*\*P<0.01 vs. vehicle group, ##P<0.01 vs. GIOP model group. GIOP, glucocorticoid-induced osteoporosis model group; ALP, alkaline phosphatase; TRAP, triiodothyronine receptor auxiliary protein.

## Results

*Celastrol reduces body weight, urine Ca/Cre, TRACP-5b, C-terminal telopeptide of type I collagen (CTX).* Body weight, urine Ca/Cre, TRACP-5b and CTX were increased in GIOP mice when compared with the vehicle group (Fig. 2). Celastrol treatment inhibited these factors when compared with the GIOP group (Fig. 2).

*Celastrol reduces ALP, TRAP and cathepsin K mRNA expression levels.* The present study quantified ALP, TRAP and cathepsin K mRNA expression levels in GIOP mice following Celastrol treatment. Fig. 3 demonstrated that ALP, TRAP and cathepsin K mRNA expression levels in GIOP mice were higher compared with the vehicle group. Treatment with Celastrol significantly reduced ALP, TRAP and cathepsin K mRNA expression levels compared with the GIOP group (Fig. 3).

*Celastrol inhibits osteocalcin, BMP-2, type I collagen, Runx-2 mRNA expression levels.* Osteocalcin, BMP-2, type I collagen, Runx-2 mRNA expression levels were determined in GIOP mice after Celastrol treatment. There was significant inhibition of osteocalcin, BMP-2, type I collagen, runx-2 mRNA expression in GIOP mice, compared with the vehicle group, which was significantly reversed in the group which received Celastrol treatment compared with the GIOP group (Fig. 4).

*Celastrol reduces PGE-2 and caspase-3 protein expression levels.* The mechanism of Celastrol on PGE-2 and caspase-3 protein expression was investigated using western blot analysis. PGE-2 and caspase-3 protein expression levels in GIOP mice were higher compared with the vehicle group (Fig. 5). Treatment with Celastrol significantly reduced PGE-2 and caspase-3 protein expression levels when compared with the GIOP group (Fig. 5).

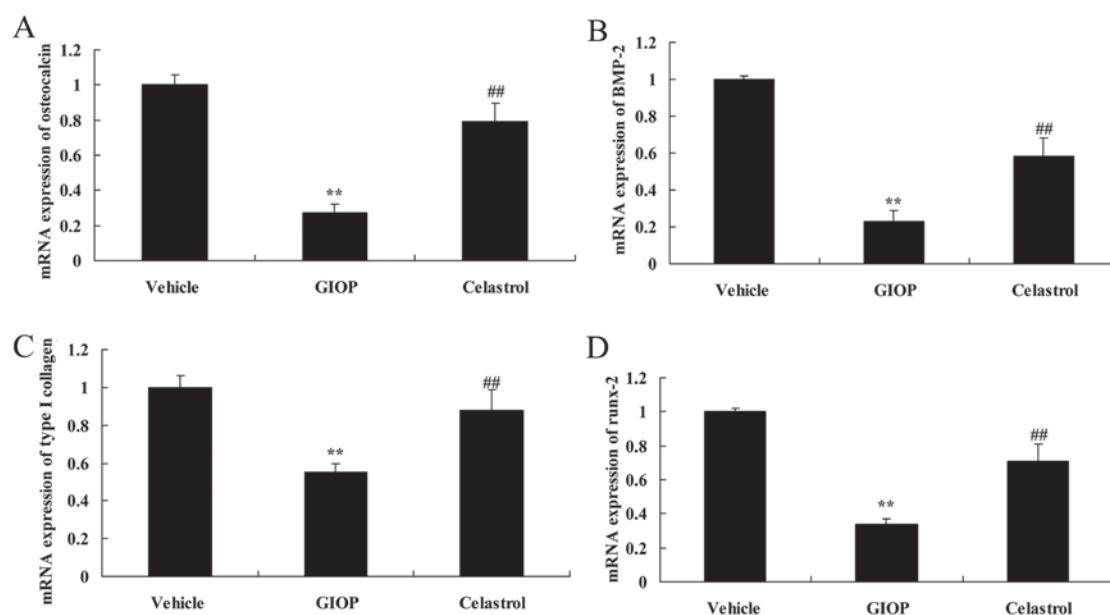


Figure 4. Celastrol induces osteocalcin, BMP-2, type I collagen and runx-2 mRNA expression levels. Celastrol induced (A) osteocalcin, (B) BMP-2, (C) type I collagen and (D) Runx-2 mRNA expression. \*\*P<0.01 vs. vehicle group; ##P<0.01 vs. GIOP model group. GIOP, glucocorticoid-induced osteoporosis model group; BMP-2, bone morphogenetic protein 2.

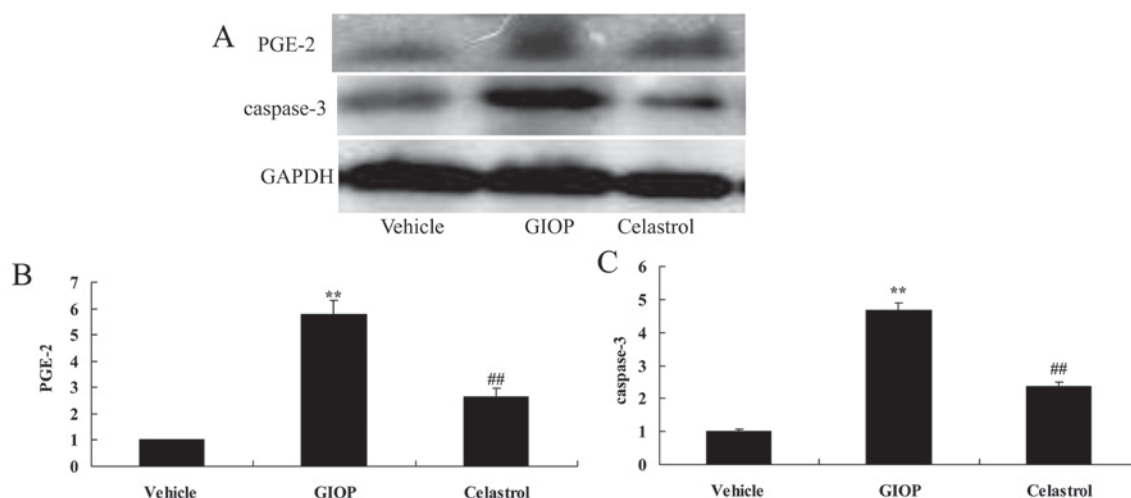


Figure 5. Celastrol inhibits PGE-2 and caspase-3 protein expression levels. PGE-2 and caspase-3 protein expression was determined using (A) western blot analysis and statistical analysis of (B) PGE-2 and (C) caspase-3 protein expression levels. \*\*P<0.01 vs. vehicle group, ##P<0.01 vs. GIOP model group. GIOP, glucocorticoid-induced osteoporosis model group; PGE-2, prostaglandin E2.

*Celastrol increases PI3K, p-AKT and p-GSK-3 protein expression levels.* To test the anti-apoptotic mechanism of Celastrol on osteoporosis, PI3K, p-AKT and p-GSK-3 protein expression levels were measured using western blot analysis. The results of western blot analysis showed that PI3K, p-AKT and p-GSK-3 protein expressions were significantly suppressed in GIOP mice compared with the vehicle group (Fig. 6). Celastrol treatment significantly increased PI3K, p-AKT and p-GSK-3 protein expression levels when compared with the GIOP group (Fig. 6).

*Celastrol increases Wnt and  $\beta$ -catenin protein expression levels.* The underlying molecular mechanism by which Celastrol regulates osteoporosis was subsequently investigated.

It was determined that Wnt and  $\beta$ -catenin protein expression levels were significantly inhibited in GIOP mice compared with the vehicle group (Fig. 7). Treatment with Celastrol significantly increased Wnt and  $\beta$ -catenin protein expression levels in GIOP mice (Fig. 7).

## Discussion

Osteoporosis is a systemic bone disease characterized by low bone mass, damaged bone microstructure, increased fragility and susceptibility to fracture (14). Minor trauma in osteoporosis patients may lead to development of fractures, disability or death; therefore, osteoporosis has become one of the primary factors affecting the quality of life of the elderly (15). Estrogen

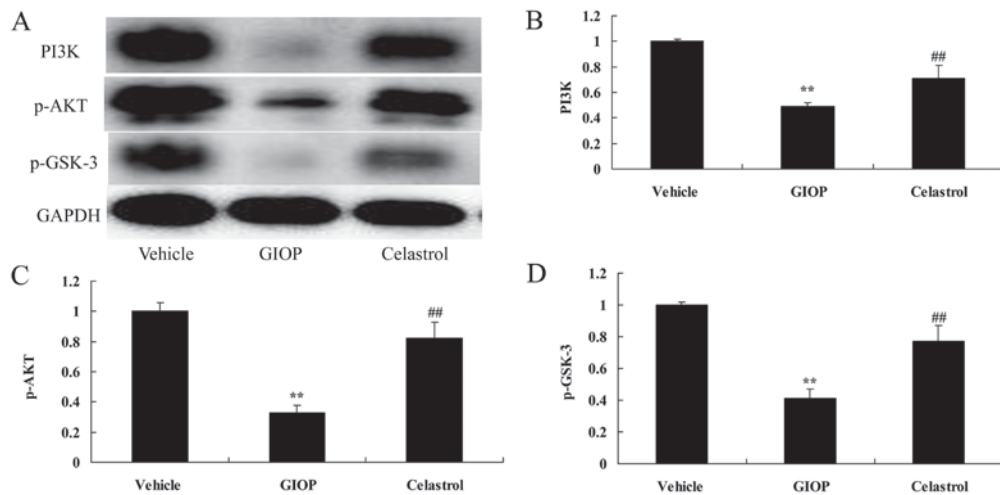


Figure 6. Celastrol induces PI3K, p-AKT and p-GSK-3 protein expression levels. PI3K, p-AKT and p-GSK-3 protein expression levels were determined using (A) western blot analysis and statistical analysis of (B) PI3K, (C) p-AKT and (D) p-GSK-3 protein expression was performed. \*\*P<0.01 vs. vehicle group, ##P<0.01 vs. GIOP model group. GIOP, glucocorticoid-induced osteoporosis model group; PI3K, phosphoinositol 3-kinase; p-AKT, phosphorylated-protein kinase B; p-GSK-3, phosphorylated-glycogen synthase kinase-3.

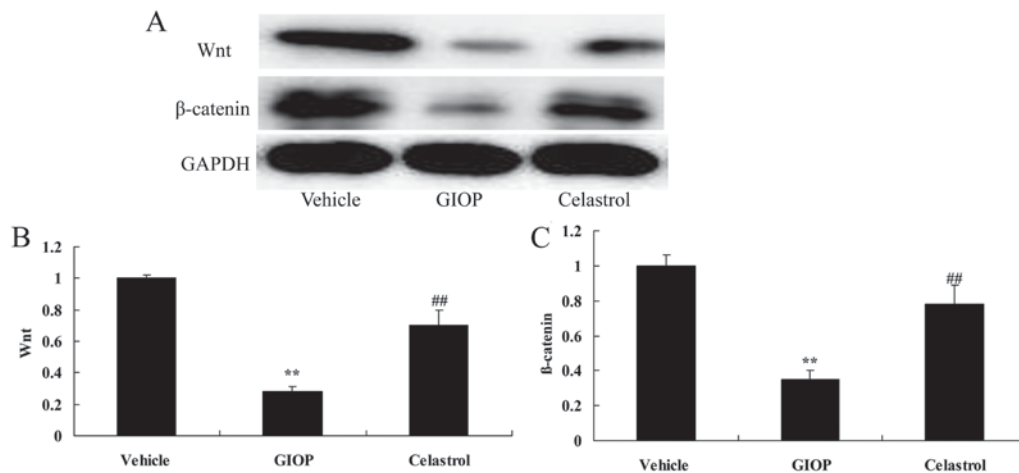


Figure 7. Celastrol induces Wnt and β-catenin protein expression levels. Wnt and β-catenin protein expression was determined using (A) western blot analysis and statistical analysis of (B) Wnt and (C) β-catenin protein expression was performed. \*\*P<0.01 vs. vehicle group, ##P<0.01 versus GIOP model group. GIOP, glucocorticoid-induced osteoporosis model group.

deficiency-induced bone loss is believed to be the primary cause of elderly osteoporosis (15). To the best of our knowledge the present study may provide the first evidence in examining whether Celastrol inhibits body weight, osteoporosis and PGE-2 and caspase-3 protein expression levels in GIOP mice.

Under normal circumstances, the PI3K/AKT signaling pathway selectively affects the physiological function of osteoblasts and osteoclasts and is activated by oxidative stress. The PI3K/AKT pathway acts on specific target genes, such as forkhead transcription factor (FOXO) and GSK-3β, to reduce the oxidative damage of osteoblasts and osteoclasts (10). Previous studies have revealed that insulin, insulin growth factor and other growth factors activate the PI3K/AKT signaling pathway, selectively regulating Wnt, FOXO, BMP and RANKL, and other signaling pathways, affecting the formation and differentiation of osteoblasts and osteoclasts and their functions, to regulate bone mass and bone strength (9,16). Therefore, as the center regulating the function of osteoblasts and osteoclasts,

the PI3K/AKT signaling pathway has an important role in maintaining the dynamic equilibrium of bone tissues under normal physiological stimulation and pathological conditions and PI3K/AKT may be a target for the treatment of osteoporosis (16). Shrivastava *et al* (17) demonstrated that Celastrol induced apoptosis in breast cancer via the PI3K/AKT pathway. The present study revealed that Celastrol significantly induced PI3K, p-AKT and p-GSK-3 protein expression levels in GIOP mice. This data suggested that Celastrol may have a significant effect on the suppression of bone cell apoptosis in GIOP mice via the PI3K/AKT pathway.

The Wnt signaling pathway regulates the growth, development, illness, aging and mortality (18). In the Wnt signaling pathway, activation of Wnt leads to the phosphorylation of the signaling molecule β-catenin and its accumulation in the nucleus, which interacts with T cytokine/lymphoid enhancement factor to mediate Wnt-induced gene transcription, and guide the differentiation of bone marrow mesenchymal stem cells into

osteoblasts (6,19). In bones, the Wnt/ $\beta$ -catenin pathway is vital for osteogenic differentiation and  $\beta$ -catenin may bind to nuclear transcription factors after entering the nucleus, to regulate a variety of proteins associated with osteogenic differentiation (5). BMP-2, a subtype of the transforming growth factor- $\beta$  superfamily, has an important role in bone formation and bone metabolic balance in adults (20). C2C12 cells may differentiate from muscle cells into osteoblasts under the continuous stimulation of BMP-2 (21). A previous study has revealed that  $\beta$ -catenin is vital to osteogenic differentiation and is downstream of the Wnt/ $\beta$ -catenin pathway and regulates osteogenic differentiation via the Wnt autocrine loop (22). The present study demonstrated that treatment with Celastrol significantly promoted Wnt and  $\beta$ -catenin protein expression levels in GIOP mice. Lin *et al* (12) previously reported that Celastrol ameliorates ulcerative colitis-associated colorectal cancer through  $\beta$ -catenin expression. The findings of the present study are consistent with previous finding (12) regarding the role of Celastrol as an effective activator of the Wnt/ $\beta$ -catenin pathway in GIOP mice and has a protective effect.

In conclusion, Celastrol treatment reduced body weight, prevented osteoporosis and inhibited PGE-2 and caspase-3 protein expression levels in GIOP mice via the PI3K/AKT and Wnt signaling pathways. The present study in conjunction with previously published findings, suggested that Celastrol may be a potential therapeutic drug against osteoporosis in the clinic.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

XL designed the study; JX, QL, YW, JL, LG and GW performed the experiments; JX and XL analyzed the data; XL wrote the manuscript.

## Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Department of Minimally Invasive Spine Surgery, The 309th Hospital of the People's Liberation Army (Beijing, China).

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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