Original Article Protective effects of naringin on glucocorticoid-induced osteoporosis through regulating the PI3K/Akt/mTOR signaling pathway

Xingtao Ge¹, Gang Zhou²

¹Department of Orthopedics 4, Rizhao People's Hospital, Rizhao, Shandong Province, China; ²Department of Orthopedic Surgery, Hainan General Hospital, Haikou, Hainan Province, China

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Abstract: Objective: To investigate the protective effects of Naringin on glucocorticoid-induced osteoporosis (GIOP) through the PI3K/AKT/mTOR signaling pathway in vivo and in vitro. Methods: Osteoblasts were cultured from the differentiated bone marrow mesenchymal stem cells (BM-MSCs) and were grouped as follows: the PBS group (the control group), the model group (Dexamethasone intervention), the LY294002 group (PI3K/AKT/mTOR pathway inhibitor intervention), the Naringin group (Naringin intervention), and the LY294002+ Naringin intervention group. Cell proliferation and differentiation were detected through cell counting kit-8 (CCK8) assay and alkaline phosphatase (ALP) staining, respectively. The formation of autophagosome was observed by Monodansylcadaverine (MDC) staining. Expressions of signaling pathway and autophagy related factors such as Beclin-1 and p62 were detected by qRT-PCR and western blot. Then, the rats were grouped as the PBS group (normal rats injected with PBS), the model group (GIOP rats injected with dexamethasone), the LY294002 group (GIOP rats injected with PI3K/AKT/ mTOR pathway inhibitor LY294002), the Naringin group (GIOP rats injected with Naringin) and the LY294002+ Naringin group (GIOP rats injected with PI3K/AKT/mTOR pathway inhibitor LY294002 and Naringin). Bone mineral density and bone histomorphometry parameters of rats in each group were compared. In addition, the expressions of pathway and autophagy related factors in cartilage tissue of rats in each groups were also detected. Results: The proliferation and differentiation abilities of osteoblasts were increased with an increasing concentration of Naringin in a dose-dependent manner. Compared with the model group, the expression of PI3K/AKT/mTOR pathway related phosphorylated proteins, the proliferation and differentiation abilities of osteoblasts, the expression of autophagosome and autophagy related factors were all increased in the Naringin group, but contrary results were found in the LY294002 group (all P<0.05). In vivo, GIOP rats had improved bone mineral density and bone morphology parameters, and elevated expressions of autophagy related factors in cartilage tissue compared to the model group through Naringin intervention, while LY294002 intervention showed the opposite effects (all P<0.05). What is more, LY294002 partially reversed the effects of Naringin on osteogenic differentiation and bone morphological parameters in GIOP. Conclusion: Naringin exerts protective effects in GIOP by the PI3K/AKT/mTOR pathway, which may be related to autophagy induction and enhanced proliferation of osteoblasts.

Keywords: Glucocorticoid-induced osteoporosis, naringin, PI3K/AKT/mTOR, osteoblasts, autophagy

Introduction

Osteoporosis is commonly found in middleaged and elderly population. It is a systemic metabolic disease accompanied by bone loss and bone tissue structure damage related to metabolic abnormalities [1]. Glucocorticoid induced osteoporosis (GIOP) is the most common type of secondary osteoporosis, which is mainly induced by diseases and drugs. Osteoblasts and osteoclasts together maintain the balance between bone formation and bone resorption. At the same time, glucocorticoids induce osteoporosis by destroying the balance between bone formation and bone resorption [2].

Osteoblasts are key cells related to bone formation, and are cultured from the differentiated bone marrow mesenchymal stem cells (BM-MSCs). Osteoblasts synthesize and secrete bone matrix, thus promoting bone formation and increasing the mass of bone. Bone formation reduction induced by suppressed osteoblast differentiation is a major pathological mechanism in the pathogenesis of GIOP [3, 4]. By inhibiting the formation of osteoclasts and enhancing the activity of osteoblasts, bisphosphonates are regarded as the most commonly used drugs for GIOP. However, many side effects were also found in medication with bisphosphonates, thus exploration for a more healthy and safe treatment scheme is urgently needed [5].

At present, natural drugs are popular research topics for various diseases. Wang et al. reported that luteolin reduced glucocorticoid-induced osteocyte apoptosis [6]. Xia et al. found that the extracts of prepared rehmannia root could prevent GIOP by inhibiting the biosynthesis of steroids [7]. Naringin is a kind of dihydroflavonoid compounds, which exists in medicinal plants or fruits. As an effective component of traditional Chinese medicine, Naringin has a variety of biological activities and pharmacological effects. Naringin has already been used for the treatment of bone diseases recently [8]. Ye et al. found that the combination of Naringin and BM-MSCs repaired articular cartilage defects of the rabbit knee joint [9]; in addition, Naringin plays an active role in the repair of large-area bone defects caused by osteosarcoma resection [10]. It has also been reported that Naringin has a good effect on the treatment of osteoporosis [11]. In conclusion, Naringin is able to inhibit osteoporosis, but the effects of Naringin in GIOP have not been clearly studied. PI3K/AKT/mTOR signaling pathway plays an important role in a variety of diseases, including GIOP [12, 13]. Zhang et al. found that the PI3K/AKT/mTOR signaling pathway regulated the proliferation, migration, and invasion of osteoblasts [14]. In addition, Yang et al. found that the inhibition of silent information regulator of transcription 1 activated the PI3K/AKT/ mTOR signaling pathway, and further enhanced the autophagy of osteoblasts, thus protecting osteoblasts [15].

In the present study, the protective effects and related mechanisms of Naringin on GIOP through the PI3K/AKT/mTOR signaling pathway were explored.

Materials and methods

Establishment of GIOP rat model

The animal experiments were conducted according to the principles of Ethics Committee of our hospital for the care and use of experimental animals. Sixty Sprague-Dawley female rats weighing 228±23 g, aged 6-7 weeks, were randomly divided into five groups. The rats in the PBS group were fed normally and were injected with PBS buffer intramuscularly (the same amount as dexamethasone sodium phosphate); the rats in the other groups were injected with dexamethasone sodium phosphate (P6950, Solarbio, Beijing, China) with the dosage of 1 mg/kg, twice a week intramuscularly to establish the GIOP model [16]. The rats were fed routinely for 8 weeks. Then, the bone mineral density was measured. After being anesthetized with pentobarbital sodium (100 mg/ kg), the rats were sacrificed and the needed tissues were taken.

Preparation of serum containing naringin

Serum containing Naringin was obtained from GIOP rats. The GIOP rats were divided into three groups and were given intragastric administration of Naringin at the doses of 40 mg/kg, 100 mg/kg, and 200 mg/kg respectively, twice a day for three consecutive days. On the 4th day, aortic blood samples were taken from the mice 1 h after the administration. Then, the rats were anesthetized and fixed on a flat plate. The abdomen was disinfected, and the skin was cut to expose the abdominal aorta. The arterial blood was collected using a blood sampling needle. After centrifugation, the serum was collected, filtered, and stored at -80°C.

Osteoblast differentiation

BM-MSCs (FS-0008) were obtained from Shanghai Fusheng Industrial Co., Ltd and were induced to differentiate into osteoblasts. The fourth generation of BM-MSCs was inoculated into 96 well plates with a density of 1*10⁴/cm². The components of inducing medium mainly include Dulbecco's modified eagle medium (DMEM) (BC-M-014, Senberg Biotechnology Co., Ltd, Nanjing, China), 10⁻⁸ mol/L dexamethasone (100129, Senberg Biotechnology Co., Ltd, Nanjing, China), 20 mL/L fetal bovine

serum (FBS, BC-SE-FBS02, Senberg Biotechnology Co., Ltd, Nanjing, China), 50 mmol/L β-sodium glycerophosphate (G9422, Sigma-Aldrich, St. Louis, Missouri, United States), 25 µL Bone morphogenetic protein (GF166, Sigma-Aldrich, St. Louis, Missouri, United States) and 50 µmol/L ascorbic acid (A5960, Sigma-Aldrich, St. Louis, Missouri, United States). We changed the inducing medium every four days. Alkaline phosphatase (ALP) staining was performed on day 15 and day 25 to identify the osteoblasts. After 30 days of culture, the morphologic changes of the cells were observed. Osteoblasts were considered as differentiated successfully when blue and purple granules appeared in the cytoplasm, and the cells were taken for follow-up study [17].

Cell culture and groupings

Osteoblasts were cultured in DMEM medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. When the confluence reached 80%-90%, the osteoblasts were inoculated into 96-well plates with a density of 4*10⁵ cells/well. Then, the cells were treated and divided into the PBS group, the model group, three Naringin groups, the LY294002 group, and the LY294002+ Naringin group. In the PBS group, the cells were placed with phosphate-buffered saline (PBS) as the control. The cells in the model group were treated with Dexamethasone (10⁻⁵ mol/L, D8040, Solarbio, Beijing, China) for 48 hours. In the 40 mg/kg Naringin group, the cells from the model group were cultured with 40 mg/kg Naringincontaining serum for 48 h; in the 100 mg/kg Naringin group, the cells from the model group were cultured with 100 mg/kg Naringincontaining serum for 48 h; in the 200 mg/kg Naringin group, the cells from the model group were cultured with 200 mg/kg Naringincontaining serum for 48 h. Based on the treatment of the model group, cells in the LY294002 group were intervened with 20 µM PI3K/AKT/ mTOR pathway inhibitor (K1020, Selleck Chemicals, United States) for 48 hours. The cells in the LY294002+ Naringin treatment group were treated with 20 µM of LY294002 and 200 mg/kg Naringin-containing serum for 48 hours.

Grouping of the animals: The PBS group (injected with PBS buffer); the model group (injected with dexamethasone intramuscularly); the

LY294002 group (the GIOP rats model injected with PI3K/AKT/mTOR pathway inhibitor LY294002 intraperitoneally); the Naringin group (the GIOP rats model took Naringin at the dose of 200 mg/kg orally); LY294002+ Naringin group (the GIOP rats model injected with LY294002 intramuscularly and took Naringin at the dose of 200 mg/kg orally). One hour after the injection of dexamethasone sodium phosphate, rats in the LY294002 group were intraperitoneally injected with PI3K/AKT/mTOR pathway inhibitor (K1020, Selleck Chemicals, United States) with the dose of 5 mg/kg/day [18]; rats in the Naringin group were given Naringin orally (S2329, Selleck Chemicals, United States) with the dose of 200 mg/kg/day [19]; rats in the LY294002+ Naringin group were intraperitoneally injected with PI3K/AKT/ mTOR pathway inhibitor and took Naringin by gavage. The rats were fed routinely and administered for 8 weeks.

Cell proliferation ability detected through cell counting kit-8 (CCK8) assay

After 48 h of continuous intervention, the cells were digested with 0.25% trypsin and seeded into 96 well plates with a density of 5×10^3 /well. The control well was also set. 24 hours later, the culture medium was changed with new DMEM medium (containing 1% FBS) and each well was added with 10 µL CCK8 solution (M4839, Adamer biology, Shanghai, China). After incubation for 4 hours, the absorbance at 450 nm was detected by multimode reader (SpectraMaxiD, Meigu molecular, Shanghai, China).

Cell differentiation ability detected through alkaline phosphatase (ALP) staining

After 48 hours of continuous intervention, cell differentiation was detected by 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP)/Nitroblue tetrazolium chloride (NBT) alkaline phosphatase staining kit (C3206, Beyotime, Shanghai, China). The treated cells were then seeded into a 96 well plate, washed with PBS for 3 times and fixed with paraformaldehyde. After washed with PBS for another 3 times, the cells were mixed with BCIP/NBT staining solution and were incubated in dark for 30 min. Then, the dye solution was discarded and the cells were washed with distilled water to terminate the reaction. The cells were incubated with nuclear

Table 1. qRT-PCR related primer sequences

Gene	Sequence 5'-3'	
PI3K	F: TGCCAAACTCTATGCCATGC	R: GGCCATCTTGGTGAAGAAGC
AKT	F: CCGCCTGATCAAGTTCTCCT	R: TTCAGATGATCCATGCGGGG
mTOR	F: ATCCAGACCCTGACCCAAAC	R: TCCACCCACTTCCTCATCTC
Beclin-1	F: ATCCTGGACCGTGTCACCATCCAGG	R: GTTGAGCTGAGTGTCCAGCTGG
p62	F: ACCCATCCACAGGTGAACTC	R: GTGGGAGATGTGGGTACAGG
GAPDH	F: CAAGGTCATCCATGACAACTTTG	R: GTCCACCACCCTGTTGCTGTAG

turation at 94°C for 30 sec; annealing at 60°C for 30 sec; extending at 68°C for 3 min; extending at 68°C for 10 min; totally 30 PCR cycles. Finally, relative expression level was quantified by formula $2^{-\Delta\Delta Ct}$, and GAPDH was used as the internal reference. The

solid red staining solution for 3 min and the reaction was observed under microscope after washing with PBS.

Secretion of autophagosomes detected by Monodansylcadaverine (MDC) staining

After 48 hours intervention, the cells were digested with 0.25% trypsin for 1 min, and were then seeded into a 6-well plate. 24 hours later, the plate with the cells was put into the incubator for 30 minutes in dark. After centrifugation. the cells were re-suspended with 1× wash buffer. Then, 90 µL cell suspension was collected and mixed with 10 µl MDC staining solution (G0170, Solarbio, Beijing, China) for 30 min in dark. The cells were centrifuged in 800 g for 5 min, washed with 1× wash buffer, and were resuspended. Cell suspension was dropped on the slide and sealed. The staining results were observed using fluorescence microscopy (at 355 nm and 512 nm) (DM4B & DM6B, Leica, Germany). According to the routine procedure, the tissues were made into paraffin sections, fixed, embedded and dehydrated, and then were stained according to the instructions of the kit.

Expression levels of PI3K, AKT, mTOR, Beclin-1 and p62 were detected by quantitative realtime polymerase chain reaction (qRT-PCR)

After 48 hours intervention, total RNA was extracted from the cells with Trizol reagent (R0016, Beyotime, Shanghai, China) and was synthesized into the first strand complementary DNA (cDNA) by adding the BeyoRT[™] II M-MLV reverse transcriptase (D7160s, Beyotime, Shanghai, China). The cDNA was then mixed with Easy-Load[™] Multiplex PCR premix (D7305s, Biyuntian, Shanghai, China) and the mixture was amplified in the PCR system (ProFlex[™], Thermo Fisher, United States). The PCR amplification procedure was set as follows: pre-denaturation at 94°C for 5 min; denaprimers were provided by Beijing B&M Biotech Co., Ltd. (China). The sequences are listed in **Table 1**. This method is also suitable for tissue experiments.

Western blot

The cells were washed with pre-cooled PBS, and were then fully lysed on ice after mixed with cell lysate. The protein samples were obtained after centrifugation (the tissue samples were cut into pieces in the buffer solution containing protease inhibitor, and were then grinded in liquid nitrogen. After centrifugation for 10 min, the supernatant was collected for subsequent experiments). The protein was quantitatively analyzed by Rapid Gold BCA Kit (A53225, Thermo Fisher, United States). The protein was separated in 10% sodium dodecyl sulfate polyacrylamide for electrophoresis and then transferred to PVDF membrane (T2234, Thermo Fisher, United States). After sealed in 5% skimmed milk for 1 hour, the membrane was incubated with primary antibodies targeting p-PI3K (1:1,000), p-AKT (1:1,000), p-mTOR (1:1,000), Beclin-1 (1:1,000), p62 (1:1,000) and GAPDH (1:1,000) at 4°C overnight; after washed with TBST for three times, the membrane was incubated with horseradish peroxidase labeled anti-rabbit secondary antibody IgG (1:2,000) for 1 hour. Then, the membrane was incubated with enhanced chemiluminescent (32209, Thermo Fisher, United States). The gray analysis of protein band was conducted using the iBright[™] FL1500gel imaging system (A44115, Thermo Fisher, United States) and Image J software. The result is shown as the ratio of gray level of target protein to internal reference GAPDH. The antibodies used above were provided by Abcam (UK).

Measurement of bone mineral density (BMD)

The BMD of rat tibia was measured by DAX small animal bone densitometer (Ranzhe

Instrument Equipment Co., Ltd., Shanghai, China). The rats were kept in supine position and the hind limbs were rotated outward. The tibia was scanned according to the instruction manual.

Morphological analysis of bone

The structure of distal tibia was measured by micro CT (SkyScan 1272, Bruker, Germany). The trabecular spacing (TS), trabecular number (TN), trabecular thickness (TT) and the ratio of bone surface to bone volume (BS/BV) were analyzed and the corresponding chart was drawn. The specific operation was carried out according to the CT operation manual.

Statistical analysis

The data were analyzed by SPSS 23.0 software. Each experiment was carried out in triplicate, and the results were presented as mean \pm standard deviation. Independent-sample t test was used to analyze the differences between two groups and one-way ANOVA combined with LSD-t test were used to compare the differences between groups. P<0.05 was considered a significant difference.

Results

Naringin enhances the proliferation and differentiation ability of osteoblasts and activates the PI3K/AKT/mTOR signaling pathway

The proliferation and differentiation ability of osteoblasts were evaluated through CCK8 assay and ALP staining assay. The results showed that the proliferation and differentiation ability of cell in the other groups were all decreased compared with the control group (all P<0.05). Besides, the proliferation and differentiation ability of cells in Naringin treatment group were enhanced compared with the model group (all P<0.05). At the same time, the proliferation and differentiation ability of cells were also enhanced with increased dosage of Naringin, and the treatment effect of 200 mg/ kg was the most optimal one (Figure 1A, 1B). In order to further explore the mechanism of Naringin on osteoblasts, qRT-PCR and western blot were used to detect the expression of PI3K/AKT/mTOR signaling pathway related genes and proteins. Results showed that the mRNA and protein expression of PI3K, AKT, and mTOR, and the mRNA and protein expression of p-PI3k, p-AKT and p-mTOR in other groups were all decreased compared with the PBS group (P<0.05). Compared with the model group, the mRNA and total protein expressions of PI3K, AKT, and mTOR in Naringin treatment group had no significant changes (all P>0.05), while the expressions of p-AKT and p-mTOR both increased (all P<0.05). Moreover, the 200 mg/ kg group had the most obvious increase (**Figure 1C-E**). These above results suggest that Naringin promotes the proliferation and differentiation of osteoblasts by activating the PI3K/ AKT/mTOR signaling pathway.

Naringin partially rescues the effect of PI3K/ AKT/mTOR signaling pathway inhibitors

PI3K/AKT/mTOR signaling pathway inhibitor was used to further explore the mechanism of Naringin on osteoblasts. Compared with the model group, the expression of signal pathway related factors in the Naringin treatment group was increased, and the proliferation and differentiation ability of cells were enhanced. Corresponding results of LY294002 group were just opposite (all P<0.05); compared with the LY294002 group, the expression of signal pathway related factors in LY294002+ Naringin group was increased, and cell proliferation and differentiation ability were also enhanced (Figure 2, all P<0.05), indicating that Naringin partially reversed the effects of PI3K/AKT/ mTOR signaling pathway inhibitor.

Naringin induces autophagy in osteoblasts

Autophagy is one of the mechanisms for maintaining the stable state of osteoblasts, so we also investigated the effects of Naringin on the formation of autophagosomes and the expression of autophagy related factors Beclin-1 and p62 in osteoblasts. Results of MDC staining, gRT-PCR, and western blot showed that, compared with the model group, the expression of Beclin-1 was increased, the expression of p62 was decreased, the positive cell rate was higher and the number of autophagy bodies was more in the Naringin treatment group. At the same time, the expression of Beclin-1 decreased, the expression of p62 increased, the positive cell rate decreased and the number of autophagosome decreased in the LY294002 group compared with the model group (all P<0.05); compared with the LY294002 group,



Figure 1. Naringin enhances the proliferation and differentiation ability of osteoblasts and activates the PI3K/AKT/ mTOR signaling pathway. A: Proliferation ability of osteoblasts was detected by CCK8 assay; B: ALP staining results (×200) and ALP activity statistics; C: Expression of total protein and phosphorylated protein of PI3K, AKT and mTOR were detected by western blot; D: The ratio of phosphorylated protein and total protein of PI3K, AKT and mTOR were detected by western blot; E: The mRNA expressions of PI3K, AKT and mTOR were detected by qRT-PCR. Compared with PBS group, *P<0.05; compared with the Model group, ^P<0.05. CCK8: cell counting kit-8; ALP: alkaline phosphatase.

the LY294002+ Naringin group had higher Beclin-1 expression, lower p62 expression,

higher positive cell rate, and more autophagosome (Figure 3, all P<0.05).



Figure 2. Naringin partially rescues the effect of PI3K/AKT/mTOR signaling pathway inhibitors. A: The proliferation ability of osteoblasts was detected through CCK8 assay; B: ALP staining results (×200) and ALP activity statistics of each group. Compared with PBS group, *P<0.05; compared with the model group, ^P<0.05; compared with the 200 mg/kg Naringin group, #P<0.05; compared with LY294002 group, &P<0.05. CCK8: cell counting kit-8; ALP: alkaline phosphatase.



Figure 3. Naringin induces autophagy in osteoblasts. A: Results of MDC staining in each group (×400); B: Statistics of positive cell rate in each group; C: The mRNA expression levels of Beclin-1 and p62 were detected by qRT-PCR; D: Protein bands were detected by western blot; E: Expression levels of Beclin-1 and p62 were detected by western



blot. Compared with PBS group, *P<0.05; compared with the Model group, ^P<0.05; compared with the 200 mg/kg Naringin group, *P<0.05; compared with LY294002 group, *P<0.05. MDC: Monodansylcadaverine.

Figure 4. Naringin improves osteoporosis in GIOP rats. A: Comparison of BMD of rats in each group; B: Comparison of TS of rats in each group; C: Comparison of TN of rats in each group; D: Comparison of TT of rats in each group; E: Comparison of BS/BV of rats in each group. Compared with PBS group, *P<0.05; compared with the Model group, ^P<0.05; compared with the 200 mg/kg Naringin group, #P<0.05; compared with LY294002 group, *P<0.05. GIOP: glucocorticoid-induced osteoporosis; BMD: Measurement of Bone mineral density.

Naringin improves osteoporosis in GIOP rats

A GIOP rat model was established, and bone mineral density and bone morphology were analyzed. The results showed that the Naringin treatment group had higher BMD, BS/BV, TT, TN values, and lower TS values than the model group, while the LY294002 group had the opposite results (all P<0.05). Compared with the LY294002 group, every index was partly reversed in the LY294002+ Naringin group, showing elevated values of BMD, BS/BV, TT and TN and decreased values of TS (**Figure 4**, all P<0.05).

Naringin promotes autophagy in rat cartilage tissues

Autophagy in the cartilage tissues of GIOP rat was detected. The results showed that the expression of Beclin-1 was increased, the expression of p62 was decreased, the positive cell rate was higher, and the number of autophagosomes was more in the Naringin treatment group compared with the model group. The expression of Beclin-1 was decreased, the expression of p62 was increased, the positive cell rate was lower, and the number of autophagosome was less in the LY294002 group compared with the model group (all P<0.05). The expression of p62 was decreased, the positive cell rate was elevated, and the number of autophagosome was less in the LY294002 group compared with the model group (all P<0.05). The expression of p62 was decreased, the positive cell rate was elevated, and the number of autophagosome was more in the LY294002+ Naringin group, compared with the LY294002 group (**Figure 5**, all P<0.05).

Discussion

Previous studies have shown that, in addition to BM-MSCs, there are also many natural drugs



Figure 5. Naringin promotes autophagy in rat cartilage tissues. A: Results of MDC staining in each group (×400); B: Positive cell rate in each group; C: mRNA expression levels of Beclin-1 and p62 were detected through qRT-PCR;

D. Protein bands were detected by western blot; E: Expression levels of Beclin-1 and p62 proteins were detected by western blot. F: The graphical abstract of the molecular mechanism in this study. Compared with PBS group, *P<0.05; compared with the Model group, ^P<0.05; compared with the 200 mg/kg Naringin group, #P<0.05; compared with LY294002 group, *P<0.05. MDC: Monodansylcadaverine.

with possible value for the treatment of GIOP [20, 21]. In this study, we explored the value of Naringin in the treatment of GIOP by exploring the protective effects of Naringin on GIOP by PI3K/AKT/mTOR signaling pathway.

Studies have found that Naringin enhances the osteogenic differentiation ability of BM-MSCs [22]: at the same time. Naringin prevents bone loss in the rat model of inflammatory bowel disease induced by glucocorticoid [23]. We first analyzed the function of Naringin at the cellular level. The results showed that the proliferation, differentiation ability and alkaline phosphatase activity of osteoblasts all increased with the increased dose of Naringin, which showed that Naringin had potential for GIOP treatment. Then we analyzed related mechanism of Naringin. Some studies reported that dexamethasone inhibited osteogenic differentiation by inhibiting PI3K/AKT signaling pathway [24, 25]; some other studies pointed out that CS-NPs promoted osteoblast differentiation by inducing autophagy through the activation of mTOR/ULK1 pathway [26]. In our study, the expressions of phosphorylated mRNA and protein related to PI3K/AKT/mTOR were increased after Naringin treatment, which showed that Naringin treatment activated the PI3K/AKT/ mTOR signaling pathway. In addition, the proliferation and differentiation ability and alkaline phosphatase activity of osteoblasts were all suppressed by pathway inhibitor, but the inhibitory effects of pathway inhibitor were partially reversed by Naringin. Therefore, we suggest that Naringin promotes osteoblast proliferation and differentiation by activating the PI3K/AKT/ mTOR signaling pathway.

Autophagy is a stress defense mechanism, which maintains the stable state of cells through the degradation of damaged cells and organelles by forming autophagosomes [27]. It has been reported that autophagy promotes the formation of osteoblasts and late osteogenesis [28, 29]. Our study found that osteoblast autophagy was promoted by Naringin and was suppressed by PI3K/Akt/mTOR pathway inhibitor. Naringin treatment partially reversed the

suppressing effects of pathway inhibitor on autophagy. Therefore, the present study demonstrated that Naringin promoted osteoblast autophagy by activating the PI3K/AKT/ mTOR signaling pathway. Larger pores in the bone and the incomplete bone structure are main characteristics of osteoporosis. GIOP rat model was constructed for the in vivo experiments. The bone mineral density measurement and micro CT showed increased BMD, BS/BV, TT, TN values and decreased TS values after Naringin treatment, which further proved the function of Naringin in GIOP. At the same time, the expression of autophagosome and related factors in GIOP rat tissues were also increased after Naringin treatment.

In conclusion, Naringin had value in the treatment of GIOP through activating the PI3K/Akt/ mTOR signaling pathway, promoting osteoblast autophagy, enhancing proliferation and differentiation, and increasing bone mineral density. However, the mechanisms of the drug are very complex. In addition to the mechanism mentioned in this present study, other mechanisms through which Naringin achieves the purpose of treatment may also exist. Thus, other mechanisms will also be further explored in combination with clinical cases in the future.

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Disclosure of conflict of interest

None.

Address correspondence to: Gang Zhou, Department of Orthopedic Surgery, Hainan General Hospital, No. 19 Xiuhua Road, Xiuying District, Haikou 570311, Hainan Province, China. Tel: +86-0898-68622210; Fax: +86-0898-68622210; E-mail: zhougang19rz@163.com

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