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Original Article

Quercetin rescued TNF-alpha-induced impairments in bone marrow-derived mesenchymal stem cell osteogenesis and improved osteoporosis in rats

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Abstract: To investigate the effect of quercetin on promoting the proliferation of bone marrow mesenchymal stem cells (BMSCs) and improving osteoporosis in rats. Rats were randomly divided into the sham, OVX and quercetin+OVX groups. In the sham and OVX groups, rats were given carboxymethyl cellulose sodium (CMC-Na). In the quercetin+OVX group, rats were given quercetin (50 mg/kg) once a day. Eight weeks after rats were treated, femurs were subjected to micro-CT scans, and bone biomechanical properties were analysed by the three-point flexural test. In addition, BMSCs were isolated and characterised by MTT, RT-PCR and Western blot analysis. In vivo, quercetin increased bone mineral density (BMD) and improved bone biomechanical properties in postmenopausal osteoporosis rat models. In vitro, TNF-α led to the activation of nuclear factor-kappa B (NF-κB) and the degradation of β-catenin, which were significantly inhibited by quercetin. Furthermore, quercetin promoted BMSC proliferation and osteogenic differentiation. In conclusion, quercetin improved in vitro models of osteoporosis and protected against TNF-α-induced impairments in BMSC osteogenesis.

Keywords: Quercetin, TNF-α, BMSCs, osteoporosis

Introduction

Postmenopausal osteoporosis (PMOP) is a systemic bone metabolism disease characterised by a progressive decrease in bone mineral density (BMD), structural deterioration, and porous bone [1-3]. Decreased oestrogen levels that occur during menopause increase a woman's risk of bone loss and osteoporosis. Currently, exogenous oestrogen is the agent of choice for the prevention and treatment of PMOP. However, long-term use of oestrogen for the treatment of osteoporosis can cause many side effects, such as the loss of mesenteric ganglion neurons and dominant ovarian follicles [4], as well as an increased incidence of cardiovascular accidents, endometrial cancer and other diseases [5-7]. Therefore, obtaining drugs with no side effects is a major goal in the treatment of osteoporosis.

Quercetin is a natural flavonoid with antioxidant and anti-inflammatory properties [8, 9]. For example, quercetin can reduce the levels of lipid peroxide and increase the ability of antioxidant systems. Quercetin also reportedly promotes the differentiation of bone marrow mesenchymal stem cells (BMSCs) into osteoblasts [10].

According to many studies, BMSCs can differentiate into osteoblasts in vitro and in vivo [11-13]. Nuclear factor-kappa B (NF-κB), a protein complex that controls transcription, cytokine production, cell survival and differentiation, plays an important role in osteogenic differentiation [14]. In clinical trials, women with PMOP exhibit increased T cell activity and elevated TNF-α production. In addition, TNF-α impairs osteogenesis through NF-κB signalling pathways in BMSCs [15].

In the present study, to find an effective treatment for PMOP, we investigated the effect of quercetin on promoting the proliferation of BMSCs and improving osteoporosis in rats.

Materials and methods

Experimental animals

Thirty adult female Sprague Dawley (SD) rats (265.70 ± 7.89 g, 8-10 weeks old) were obtained from the Hunan SJA Laboratory Animal Co., Ltd. (Changsha, China). All animal procedures were performed according to the guidelines of the Animal Care Committee of Nanchang University. All rats were housed under specific pathogen-free conditions (22°C-26°C and 40%-60% humidity) with free access to food and tap water.

SD rat in vivo experiments

All rats were randomly divided into the sham group, ovariectomy group (OVX group) and quercetin+OVX group. Ovariectomies were performed on rats in the OVX group and quercetin+OVX group under general anaesthesia with an abdominal longitudinal incision. The sham groups underwent a sham surgery in which an equal volume of fat surrounding the bilateral ovaries was removed. Three days after the operation, the sham and OVX groups were given 3 ml carboxymethyl cellulose sodium (CMC-Na, Sigma-Aldrich Corporation, St. Louis, MO) daily by oral gavage. The quercetin+OVX group was given 50 mg/kg quercetin solvent (Sigma-Aldrich Corporation, St. Louis, MO) daily by oral gavage.

Rats were weighed once a week to adjust the dosage according to rat body weight. After 8 weeks, rats were sacrificed by cervical dislocation. Each rat's left femur was scanned by micro-computed tomography (Bruker micro-CT SkyScan1127, Bruker micro-CT Corporation, Kontich, Belgium). Compared to the proximal and middle regions, the trabecular bone is rich in the distal femur; thus, the scan was performed from the proximal growth plate in the distal direction (18 μm/slice) for every selected femur sample. The region of interest was located between 1.5 and 3.5 mm distal to the growth plate epiphyseal junction. From the bottom of the growth plate, one hundred images were analysed. The BMD was measured by micro-CT. By using a Bose Electro Force Testing system (Bose Electro Force 3550, Bose Corporation, Eden Prairie, MN), bone biomechanics were analysed by the three-point bending test of each rat's right femur. Bone samples were tested with a 1 mm indenter at a speed of 0.01 mm/s with a 15 mm span (L). Force and deflection were automatically recorded [16]. The output parameters included elastic load (the force required to cause bone specimens to deform in units of N), maximum load (the maximum force that the bone can resist, N), elastic radial degree and the maximum radial degree (maximum degree of the bone displacement, mm) [17].

BMSC isolation and culture

BMSCs were flushed from femurs and tibias taken from SD rats. Cells were centrifuged at 1000 g for 5 min and suspended in Dulbecco's Modified Eagle's Medium with low glucose (DMEM, HyClone, Suwanee, GA) supplemented with 10% foetal bovine serum (FBS, HyClone, Suwanee, GA), glutamine, penicillin and streptomycin and then cultured in a humidified incubator with 5% CO_2 at 37°C. The medium was changed every 3 days. Cells used in these experiments were harvested from the third passage.

Effect of quercetin on the cellular activity of BMSCs by MTT

BMSCs were plated in 96-well plates at a concentration of 1×10^4 cells/ml and cultured with 200 μl DMEM per well. Cells were stimulated with 5 ng/mL murine TNF-α (Sigma-Aldrich Co, St Louis, MO) in the TNF-α group, treated with quercetin (1.0 μm) in the quercetin group, treated with TNF-α and quercetin in the TNF-α+quercetin group, and given no treatment in the control group. After 24 hours of incubation to allow the cells to adhere to the bottoms of the wells, 20 μl 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) solution (5 mg/ml) (Beyotime Biotechnology, Shanghai, China) was added to each well. After 4 hours of incubation, the medium in each well was discarded, a 150 μl aliquot of dimethyl sulfoxide (DMSO) was added to each well, and samples were agitated at a speed of 50 oscillations/min for 10 min. A Universal Microplate Reader (Bio-Tek, Winooski, VT) was used to measure the optical density of each well at 450 nm. Wells containing media without cells were used as blanks. All measurements were performed in triplicate.

Alizarin red staining of calcium nodule formation

BMSCs were inoculated into 96-well plates at a concentration of 1×10^4 cells/ml and cultured.

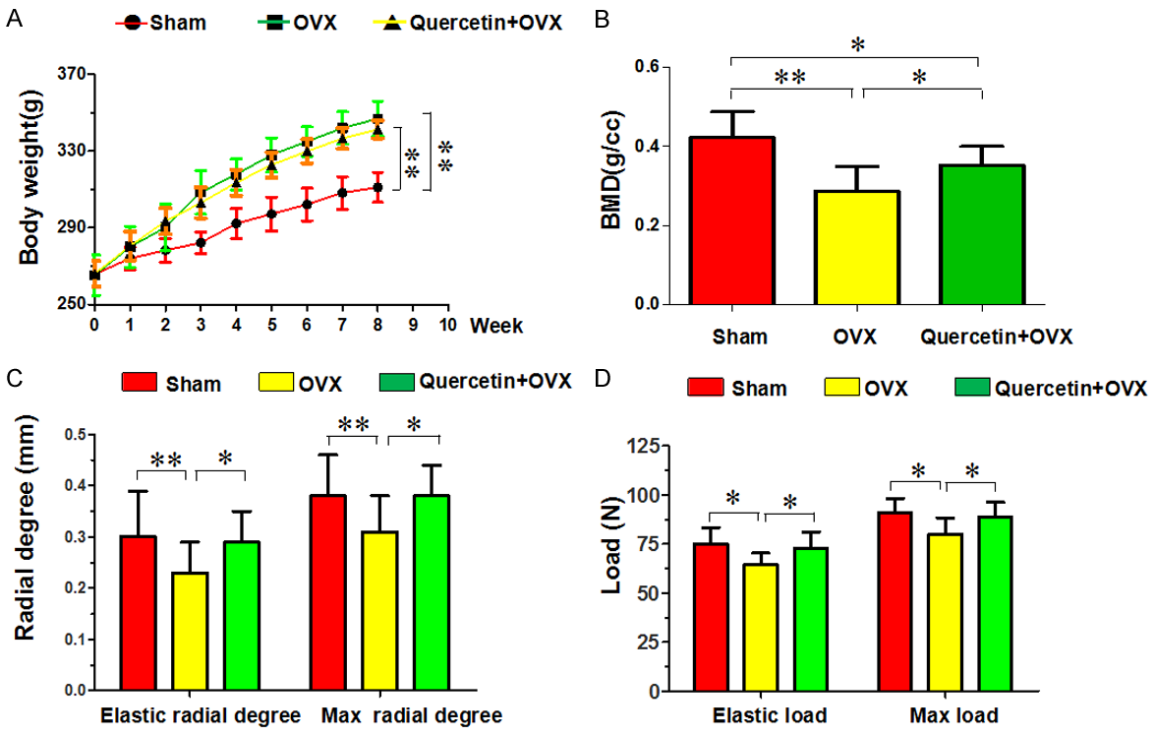

Figure 1. The effect of quercetin on body weight, BMD and bone biomechanical properties in PMOP model rats. A. The trend of weight gain in the three groups of rats. Compared with the weight of rats in the sham group, the weight of rats in the quercetin+OVX and OVX groups gradually increased after 8 weeks. Weight was not significantly different between the OVX group and quercetin+OVX group. B. The BMD value of the metaphyseal metaphysis of the rat femoral shaft was calculated by micro-CT. The BMD of the Quercetin+OVX group was higher than that of the OVX group but lower than that of the sham group. C and D. By the three-point flexural test, the biomechanical indexes of the elastic and maximum radial degrees and elastic and maximum loads of the rat femur were recorded. Compared with the sham group, the OVX group showed decreases in the elastic radial degree, maximum radial degree, elastic load and maximum load, whereas compared with the OVX group, the quercetin+OVX group showed increases in those measurements. $^{**}P<0.01$, $^{*}P<0.05$, values are presented as the mean ± standard deviation.

Cells were treated as described above and fixed with 70% ethyl alcohol. Then, 2% alizarin red (Sigma-Aldrich Co, St Louis, MO) (pH 8.3) was added, and the cells were stained at 37°C for 30 min. Mineralised calcium nodules were observed under a light microscope to detect matrix mineralisation.

Real-time PCR detection of Runx2 and Osterix expression

Cells were lysed in 1 ml TRIzol reagent (Invitrogen Corporation, Carlsbad, CA), and RNA was isolated using RNeasy mini-kits (Qiagen, Duesseldorf, Germany) according to the manufacturer's protocol. A PrimeScript™ First Strand cDNA Synthesis Kit (Takara, Otsu, Japan) was used to transcribe RNA into cDNA. Then, a FastStart Universal Probe Master (Roche Applied Science, Indianapolis, IN) was used to perform real-time polymerase chain reaction (PCR). Amplification conditions were as follows: 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. Gene expression was quantified using the standard curve method with actin as the endogenous control. RNA was collected from three separate wells for each group, and the expression levels of Runx2 and Osterix were assessed.

Western blot analysis of NF-κB and β-catenin expression

For Western blot analysis, lysates were prepared in Pro-PREPTM Protein Extraction Solution (Boca Scientific Inc., Boca Raton, FL). Samples were heated with equal volumes of sodium dodecyl sulfate (SDS) sample buffer (Invitrogen Corporation, Carlsbad, CA) and 10

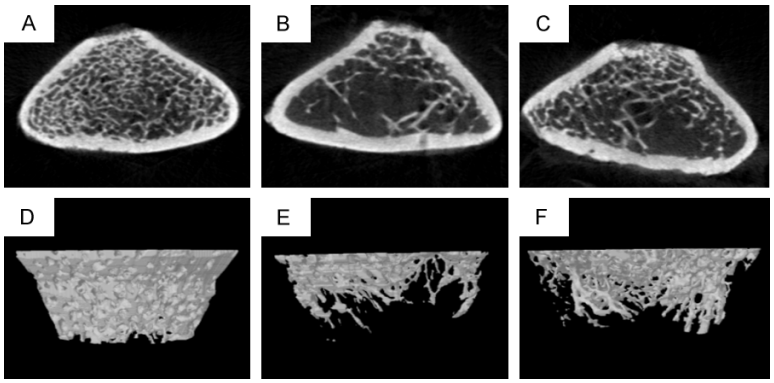

Figure 2. Positive effects of quercetin on bone micro-architecture parameters in PMOP rats. In this figure, A-C are the results of the 2D scans of the femoral diaphysis and metaphysis in the sham group, OVX group and quercetin+OVX group, respectively. D-F are the results of the 3D scans of the femoral diaphysis and metaphysis in the sham, OVX and quercetin+OVX groups, respectively. Compared with the sham group, the OVX group showed markedly decreased trabecular number and thickness in the femoral metaphysis. After treatment with quercetin, the trabecular number and thickness of the rat femoral metaphysis increased.

mM dithiothreitol (DTT, Thermo Fisher Scientific, Waltham, MA) at 95°C for 5 min. Then, 20 μg of total cell lysate protein was electrophoresed and further analysed using a standard. Membranes were treated with antibodies against the following proteins: NF-κB and β-catenin (Cell Signaling Technology, Beverly, MA), actin (Sigma-Aldrich Co, St Louis, MO) to quantify NF-κB and β-catenin, TNF-α for quercetin-induced BMSC proliferation and differentiation via the NF-κB/β-catenin signalling pathway, and goat anti-mouse immunoglobulin G-horseradish peroxidase (IgG-HRP) and goat anti-rabbit IgG-Biotin (Santa Cruz Biotechnology, Dallas, TX). HRP-conjugated goat anti-rabbit secondary antibodies (1:5,000) (Santa Cruz Biotechnology, Santa Cruz, CA) were used. Membranes were visualised using an enhanced chemiluminescence (ECL) system (Pierce, Rockford, IL), and protein expression levels were normalised to actin protein levels.

Statistical analysis

Data are presented as the mean ± standard deviation. Significant differences among groups were evaluated using SPSS 22.0 software by one-way analysis of variance (ANOVA) with repeated measures. $P<0.05$ was considered statistically significant, and $P<0.01$ indicated a significant difference.

Results

Effect of quercetin on rat body weight

After 8 weeks, rat body weight significantly gradually increased in the OVX and quercetin+OVX groups compared with that in the sham group (**Figure 1A**). There was no significant difference between the OVX and quercetin+OVX groups (**Figure 1A**).

Effect of quercetin on BMD and bone biomechanical properties

After ovariectomy, rats lacked oestrogen stimulation, resulting in decreased bone mass. The BMD of rats in the OVX group was significantly lower than that of rats in the sham group, indicating that OVX-induced osteoporosis was successfully established. The BMD of the quercetin+OVX group was higher than that of the OVX group (**Figure 1B**), suggesting that bone loss was inhibited after oral administration of quercetin to PMOP model rats. However, the BMD of rats in the quercetin+OVX group was lower than that of rats in the sham group. These results indicate that quercetin can improve BMD but cannot reverse bone loss.

Radialis elasticity, elastic load and maximum load are biomechanics-related indicators that can reflect bone biomechanical properties. To investigate the role of quercetin in improving bone function, we measured radialis elasticity, elastic load, and maximum load in the different groups. Compared with the femurs of the sham group, the femurs of the OVX group showed decreases in all these measures. However, the above indicators in the quercetin+OVX group were higher than those in the OVX group (**Figure 1C** and **1D**).

Quercetin increases trabecular number and thickness in the rat femoral metaphysis

A standard structural extraction approach was applied to micro-CT for the assessment of bone trabecular number and thickness to describe

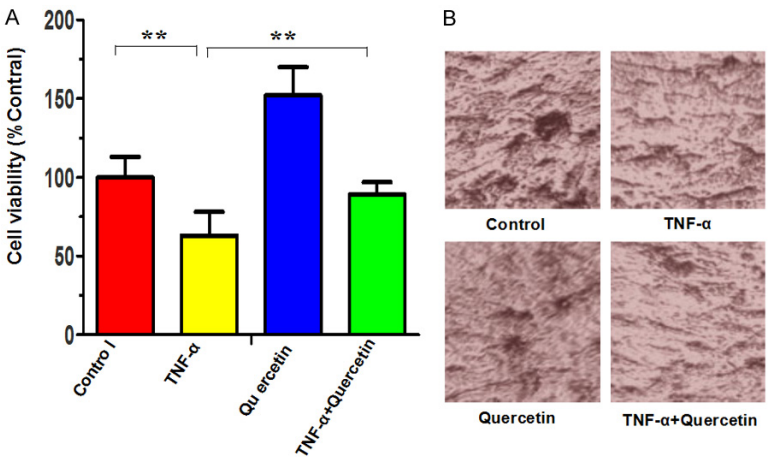

Figure 3. Effect of quercetin on the viability of BMSCs and calcium nodule formation. A. BMSCs in different groups were detected by MTT assays. Compared with the control group, the TNF-α group showed significant inhibition of the cell viability of BMSCs. The cell viability of BMSCs was higher following the addition of TNF-α and quercetin than following the addition of TNF-α alone; however, there was no significant difference compared with the control. B. The formation of calcium nodules was detected by alizarin red staining. The number of calcium nodules in the TNF-α group was significantly lower than that in the control group. The calcium nodules of the TNF-α and quercetin group were significantly higher than those of the TNF-α group. Compared with the other three groups, the quercetin group showed a significant increase in calcium nodule formation. $^{**}P<0.01$, $^{*}P<0.05$, values are presented as the mean ± standard deviation.

trabecular bone morphology and explain changes in bone mass. Compared with the sham group, the OVX group showed a significantly reduced trabecular number at the femoral metaphysis, and trabecular thickness was diminished. After treatment with quercetin, the trabecular number and thickness of the rat femoral metaphysis were increased (**Figure 2**).

Quercetin weakens the inhibitory effect of TNF-α on the activity of BMSCs

To investigate the protective roles of quercetin, cellular viability was detected by MTT assays. Compared with control medium, medium containing TNF-α caused a significant reduction in the cell viability of BMSCs. Compared with the addition of medium containing TNF-α alone, the addition of medium containing both quercetin and TNF-α solvent increased the cell viability of BMSCs, and there was no significant difference compared with the control (**Figure 3A**).

Quercetin promotes calcium nodule formation

To investigate the role of quercetin in the osteogenic differentiation of BMSCs, we observed calcium nodule formation in the four groups by alizarin red staining. The number of calcium nodules in the TNF-α group was significantly lower than that in the control group. The combination of quercetin and TNF-α increased the number of calcium nodules. Compared with the other groups, the quercetin group showed a significant increase in calcium nodule formation (**Figure 3B**).

Quercetin reduces the inhibitory effect of TNF-α on Runx2 and Osterix

Runx2 and Osterix are the master transcription factors in bone formation [18]. To investigate the effect of quercetin on the osteogenic differentiation of BMSCs, we detected Runx2 and Osterix using RT-PCR. Compared with the control group, the TNF-α group showed significantly lower expression levels of Runx2 and Osterix. The expression of these two transcription factors was higher in the quercetin group than in the TNF-α group (**Figure 4A**, **4B**).

Effect of quercetin on the NF-κB/β-catenin signalling pathway

NF-κB can promote β-catenin degradation and inhibit osteogenic differentiation of BMSCs. To investigate the effect of quercetin on the NF-κB/β-catenin signalling pathway, we observed the protein expression content and grey ratio. TNF-α activated NF-κB, increased the expression of NF-κB protein and promoted the degradation of β-catenin protein. After treatment with TNF-α and quercetin, the expression of NF-κB protein was decreased, and the expression of β-catenin protein was increased (**Figure 4C** and **4D**).

Discussion

Worldwide, osteoporosis is a serious problem that results in fractures, which lead to disability and high costs to society. Though oestrogen

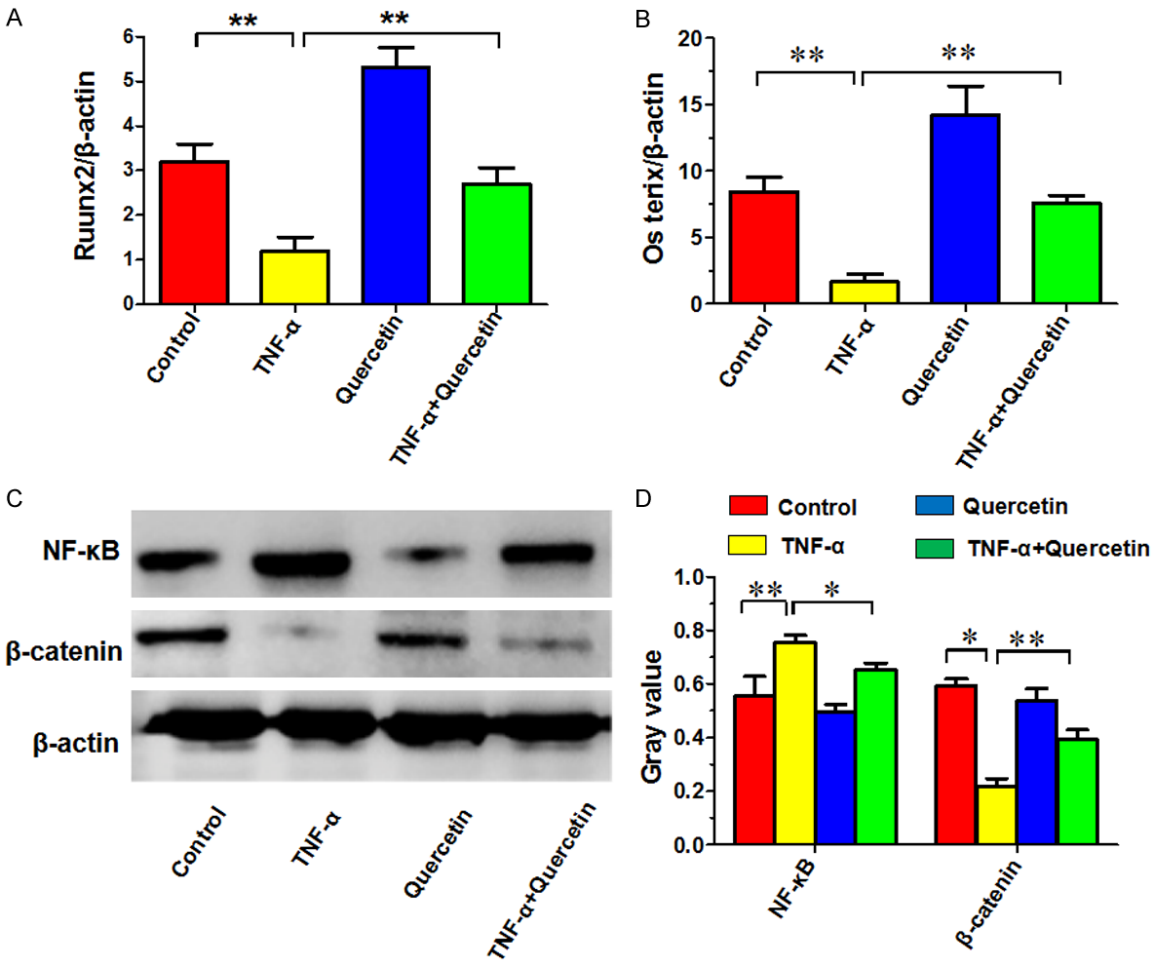

Figure 4. The effect of quercetin on the formation of bone and NF-κB/β-catenin signalling pathways. A and B. Real-time PCR was used to detect the expression of two transcription factors, Runx2 and Osterix. Compared with the control group, the TNF-α group showed significantly decreased expression levels of Runx2 and Osterix. Compared with the TNF-α group, the TNF-α and quercetin group showed increased expression of these two transcription factors. C. Western blot analysis of NF-κB and β-catenin. D. The expression levels of NF-κB and β-catenin proteins were converted to grey values. Compared with the control group, the TNF-α group showed increased expression of NF-κB protein; however, β-catenin protein was decreased. Compared with the TNF-α group, the TNF-α and quercetin group showed decreased expression of NF-κB protein, whereas the expression of β-catenin protein was increased. $^{**}P<0.01$, $^{*}P<0.05$, values are presented as the mean ± standard deviation.

replacement therapy was once the only approved osteoporosis medication, it is not as widely used today. In a previous study, we found that the extract of *Eucommia ulmoides* leaves could be used to treat osteoporosis [19]. Quercetin is the main component of *Eucommia ulmoides* leaf extract; thus, we speculated that quercetin could be used in the treatment of osteoporosis.

Quercetin is a major component of Eucommia flavonoids [20] and exerts both anti-inflammatory and antioxidative effects [21-23]. Here, we observed the effect of quercetin on PMOP rats; quercetin increased the trabecular number, trabecular thickness, and bone mineral content of ovariectomised rats without affecting their body weight. Thus, quercetin can improve bone micro-structure and improve bone quality. Our bone biomechanical analysis further showed that quercetin can increase the load and elasticity of bone, indicating that quercetin can improve bone function. Therefore, quercetin can treat PMOP in terms of both structure and function.

BMSCs are an important osteogenic precursor cell that can differentiate in an osteogenic

direction and play an important role in bone development, remodelling, repair, and regeneration [24, 25]. In PMOP rats, continuous increases in inflammatory factors, especially chronic, sustained increases in TNF-α, will lead to decreased osteogenic differentiation and increased BMSC apoptosis [26, 27]. Significant reductions in BMSCs, proliferation and osteogenic differentiation are important causes of osteoporosis [28, 29]. Our results also confirmed that TNF-α can inhibit the activity of BMSCs, reduce mineralisation, and decrease the expression of osteogenesis-related transcription factors Runx2 and Osterix. More importantly, our results showed that TNF-α induced BMSC osteogenic differentiation and that quercetin antagonised TNF-α. These findings indicated that quercetin enhances the activity and function of BMSCs that are closely related to improvements in osteoporosis.

NF-κB, a central regulator of inflammatory mediators in cells, is involved in regulating the immune response, inflammatory response, cell proliferation, and tumourigenesis [30-32]. TNF-α activates NF-κB, and activated NF-κB can reduce the expression of bone formation-associated proteins Runn2, Sp7, Alp, and Ocn, thereby reducing BMSC-mediated bone formation [33]. Our results also confirmed that TNF-α could increase the protein expression of NF-κB and inhibit osteogenic differentiation. A large number of studies have confirmed an interaction between the Wnt/β-catenin signalling pathway and NF-κB signalling pathway, and NF-κB can promote the degradation of β-catenin [34, 35]. β-catenin, a multifunctional protein that can mediate cell adhesion and participate in gene expression, plays an important role in regulating the proliferation and differentiation of osteoblasts [36-38]. Therefore, we also examined the expression of β-catenin and found that the degradation of β-catenin increased in the presence of TNF-α. In a previous study, Chang and colleagues used different doses of TNF-α to treat BMSCs in vitro to detect the protein expression of P65 and IκB. These authors confirmed that TNF-α inhibits osteogenic differentiation of BMSCs by activating NF-κB and that activated IKK induction of Smurf1 and Smurf2 promotes β-catenin degradation [39]. Our findings are consistent with the results obtained by Chang and colleagues, indicating that TNF-α can promote the degradation of β-catenin by activating the expression of NF-κB and reducing the activity and osteogenic function of BMSCs. Interestingly, we also found that quercetin can inhibit the activation of NF-κB by TNF-α, thereby reducing the degradation of β-catenin and increasing osteogenic differentiation. Therefore, we believe that quercetin inhibits the activation of the NF-κB/β-catenin signalling pathway by inhibiting TNF-α, thereby increasing the activity and osteogenic differentiation of BMSCs.

Conclusion

Quercetin can improve bone structure and function in PMOP rats and enhances BMSC activity and osteogenic differentiation ability. Thus, quercetin is a possible mechanism for antagonising the TNF-α-induced NF-κB/β-catenin signalling pathway.

Acknowledgements

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Disclosure of conflict of interest

None.

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