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Galangin inhibits lipopolysaccharide-induced inflammation and stimulates osteogenic differentiation of bone marrow mesenchymal stem cells via regulation of AKT/mTOR signaling

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Abstract

Background: Bone marrow mesenchymal stem cells (BMSCs), with the abilities of multidirectional differentiation and self-renewal, have been widely used in bone repair and regeneration of inflammation-stimulated oral diseases. Galangin is a flavonoid isolated from *Alpinia officinarum*, exerts anti-obesity, antitumor, and anti-inflammation pharmacological effects. The roles of galangin in lipopolysaccharide-induced inflammation and osteogenic differentiation of BMSCs were investigated.

Methods: BMSCs were isolated from rat bone marrow and identified by flow cytometry. The isolated BMSCs were treated with 1 µg/mL lipopolysaccharides or cotreated with lipopolysaccharides and different concentrations of galangin. Cell viability and apoptosis were detected by MTT (tetrazolium component) and flow cytometry. ELISA was used to detect inflammation. Alizarin red staining was used to investigate osteogenic differentiation.

Results: The rat BMSCs showed negative rate of CD34, and positive rate of CD29 and CD44. Lipopolysaccharides treatment reduced cell viability of BMSCs, and promoted the cell apoptosis. Incubation with galangin enhanced cell viability of lipopolysaccharide-stimulated BMSCs, and suppressed the cell apoptosis. Galangin decreased levels of TNF-α, IL-18, and IL-6 in lipopolysaccharide-stimulated BMSCs through down-regulation of NF-κB phosphorylation (p-NF-κB). Galangin up-regulated expression of osteo-specific proteins, collagen type I alpha 1 (COL1A1), osteopontin (OPN), and runt-related transcription factor 2 (RUNX2), to promote the osteogenic differentiation of lipopolysaccharide-stimulated BMSCs. Protein expression of p-AKT and p-mTOR in lipopolysaccharide-stimulated BMSCs were increased by galangin treatment.

Conclusion: Galangin exerted an anti-inflammatory effect against lipopolysaccharide-stimulated BMSCs and promoted osteogenic differentiation through the activation of AKT/mTOR signaling.

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Introduction

Immuno-inflammatory oral diseases, including periodontitis, peri-implantitis, and periodontitis are caused by microbial infections and characterized by the destruction and irreversible loss of the alveolar bone and loosening and loss of teeth.¹ Immuno-inflammatory oral diseases with high prevalence are generally treated with surgical or non-surgical management.² Repair of the alveolar bone loss is considered a promising strategy for the treatment of immuno-inflammatory oral diseases.³ Bone marrow mesenchymal stem cells (BMSCs), with the abilities of multidirectional differentiation and self-renewal, have been widely used in periodontal regeneration.⁴

Oral bacteria, *Porphyromonas gingivalis*, with the ability to secrete virulence factors, such as lipopolysaccharides, have been considered an important risk factor in the immuno-inflammatory oral disease.⁵ Lipopolysaccharides have been shown to induce the secretion of osteoclastic cytokines, TNF- α , IL-1 β , and receptor activator of nuclear factor κ B ligand, in various cell types, including BMSCs.⁶ Moreover, lipopolysaccharides also impair osteoblast differentiation and suppress osteogenic differentiation tendency of BMSCs.⁷ Suppression of lipopolysaccharide-stimulated inflammation and differentiation in BMSCs contributed to the amelioration of immuno-inflammatory oral diseases.⁸

Galangin is a natural flavonoid extracted from *Alpinia officinarum* with pharmacological activities, such as anticancer, antibacterial, anti-inflammatory, hepatoprotective, and antiarthritic effects.⁹ For example, galangin inhibited cell proliferation and metastasis of osteosarcoma, and promoted cell apoptosis.¹⁰ Galangin induced differentiation of Th cells into immunosuppressive Tregs, and conferred tolerogenic property of dendritic cell in response to lipopolysaccharides.¹¹ Galangin also inhibited lipopolysaccharide-stimulated microglial activation and suppressed the inflammatory response.¹² In orthopedic research, galangin protected osteoblast against zoledronate-induced cell death,¹³ and suppressed osteoclast formation and inflammation in bone marrow-derived macrophages.¹⁴ Moreover, galangin promoted alkaline phosphatase activity to induce osteogenic differentiation in osteosarcoma cells.¹⁵ Therefore, it was hypothesized that galangin might inhibit inflammation of lipopolysaccharide-stimulated BMSCs, and promote the osteogenic differentiation. The effects of galangin on inflammation and osteogenic differentiation of lipopolysaccharide-stimulated BMSCs, as well as the related mechanism, were determined in this study.

Materials and Methods

Isolation and culture of BMSCs

Male Sprague-Dawley rats (4 weeks old and 120-150 g weight) were purchased from Slac Laboratory Animal Corp. (Shanghai, China). Rats were sacrificed using cervical dislocation, and the tibia and femur bones were carefully harvested from the rats. Eye scissors were used to intersect the terminals of bones, and the bone marrow was collected followed by washing with complete α -minimum essential medium (Invitrogen, Carlsbad, CA, USA). The cell suspensions were

then seeded and cultured in the medium with 10% fetal bovine serum (Invitrogen) at 37°C in an incubator containing a 5% CO₂ atmosphere. Twenty-four hours later, the nonadherent cells were removed and the isolated BMSCs were cultured in 10-cm cell culture dishes. Cells at passages 3-5 were used for the functional assays. All animal experiments were approved by the Ethics Committee of The Sixth Affiliated Hospital of Xinjiang Medical University for the use of animals and conducted in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines.

BMSCs identification

The isolated BMSCs (passages 3-5) were harvested by incubation with 0.05% Trypsin (Sigma-Aldrich, St Louis, MO, USA). Cells were resuspended in a 100- μ L phosphate-buffered saline solution (Beyotime, Beijing, China) and stained with fluorescent isothiocyanate (FITC)-anti-rat CD34 (Abcam, Cambridge, UK), phycoerythrin-anti-rat CD29 and CD44 (Abcam) antibodies. Cells were then analyzed by the FACS flow cytometer (Life Technologies, Darmstadt, Germany) for identifying BMSCs.

Cell viability and apoptosis assays

BMSCs were seeded in 96-wells plates and treated with 1 μ g/mL of lipopolysaccharides (Sigma-Aldrich) or cotreated with lipopolysaccharides and different concentrations of galangin (5, 10, or 20 μ M; Sigma-Aldrich) for 24 h. The plates were then added with a 5 mg/mL MTT solution (10 μ L) (Beyotime) and incubated for another 4 h. Dimethyl sulfoxide was added into each well, and the absorbance at 450 nm was measured by microplate reader (Bio-Rad, Hercules, CA, USA). For cell apoptosis analysis, BMSCs post lipopolysaccharides or galangin treatment were harvested using trypsin and then resuspended in binding buffer of Annexin V FITC/propidium iodide (PI) staining kit (Invitrogen). Cells were stained with 5 μ L of PI and 5 μ L of FITC-annexin V (Invitrogen). The apoptotic ratio was calculated using the FACS flow cytometer (Life Technologies).

ELISA

The cultured medium of BMSCs were collected, and the BCA kit (Applygen, Beijing, China) was used to determine the protein concentration. Levels of TNF- α , IL-6, and IL-1 β were determined via the ELISA kits (ExCell Biology, Inc., Shanghai, China).

Alizarin Red staining

BMSCs in osteogenic medium (90% DMEM, 10% fetal bovine serum, 0.25 mM ascorbate, 10 mM β -glycerolphosphate, and 0.1 μ M dexamethasone; HyClone, Logan, UT, USA) were seeded in 6-wells plates, and treated with 1 μ g/mL lipopolysaccharides (Sigma-Aldrich) or cotreated with lipopolysaccharides and different concentrations of galangin (5, 10 or 20 μ M; Sigma-Aldrich). The medium was changed every 3 days, and 2 weeks later, the cells were fixed in 4% paraformaldehyde and incubated with Alizarin Red (Sigma-Aldrich).

The BMSCs were photographed under microscope (Olympus, Tokyo, Japan), and the absorbance at 620 nm was measured by the microplate reader (Bio-Rad).

Western blotting

BMSCs were lysed in RIPA buffer (Beyotime), the isolated protein samples were separated by 10% SDS-PAGE, and then transferred onto nitrocellulose membranes. The membranes were blocked in 5% BCA, and then incubated with specific antibodies: anti-NF- κ B and anti-p-NF- κ B (1:1500, Abcam), anti-COL1A1 and anti-OPN (1:2000, Abcam), anti-RUNX2 and anti- β -actin (1:2500, Abcam), anti-AKT and anti-p-AKT (1:3000, Abcam), anti-mTOR and anti-p-mTOR (1:3500, Abcam) overnight. Following a wash with phosphate buffered saline, the membranes were then probed with horseradish peroxidase-conjugated secondary antibody (1:4000, Abcam). The immunoreactivities were visualized using enhanced chemiluminescence (Sigma-Aldrich) followed by incubation with tetramethylbenzidine.

Statistical analysis

All data with at least triplicates were expressed as mean \pm SEM, and analyzed by the student's *t* test or one-way analysis of variance under SPSS software. A *p* value <0.05 was considered as statistically significant.

Results

Galangin suppressed cell apoptosis of lipopolysaccharide-treated BMSCs

BMSCs were isolated from the rats, and flow cytometry showed that the isolated BMSCs had a negative rate of

CD34 <2%, positive rate of CD29 and CD44 >95% (Figure 1A), indicating the high purity of BMSCs. BMSCs were treated with lipopolysaccharides, and the cell viability of BMSCs was decreased (Figure 1B), while the cell apoptosis was increased (Figure 1C) by lipopolysaccharides. However, incubation with galangin increased cell viability of lipopolysaccharide-treated BMSCs (Figure 1B) and reduced the cell apoptosis (Figure 1C) in a dosage-dependent manner, suggesting the anti-apoptotic effect of galangin against lipopolysaccharide-treated BMSCs.

Galangin suppressed lipopolysaccharide-induced inflammation in BMSCs

Lipopolysaccharides treatment induced up-regulation of TNF- α , IL-6, and IL-1 β , thus promoting the inflammatory response in BMSCs (Figure 2A). However, galangin reduced the levels of TNF- α , IL-6, and IL-1 β of lipopolysaccharide-treated BMSCs in a dosage dependent way (Figure 2A). Moreover, galangin attenuated lipopolysaccharide-induced increase of p-NF- κ B in BMSCs (Figure 2B), suggesting that galangin suppressed activation of NF- κ B signaling to reduce lipopolysaccharide-induced inflammation in BMSCs.

Galangin promoted osteogenic differentiation of lipopolysaccharide-treated BMSCs

Osteoblast differentiation markers, COL1A1, OPN and RUNX2, in BMSCs, were reduced by lipopolysaccharides treatment (Figure 3A). Galangin enhanced expression of COL1A1, OPN and RUNX2 in lipopolysaccharide-treated BMSCs (Figure 3A) to stimulate the osteogenic differentiation. Alizarin Red staining demonstrated that the mineralization of BMSCs was reduced by lipopolysaccharides (Figure 3B), while galangin enhanced the mineralized nodule formation in lipopolysaccharide-treated BMSCs (Figure 3B), revealing that galangin

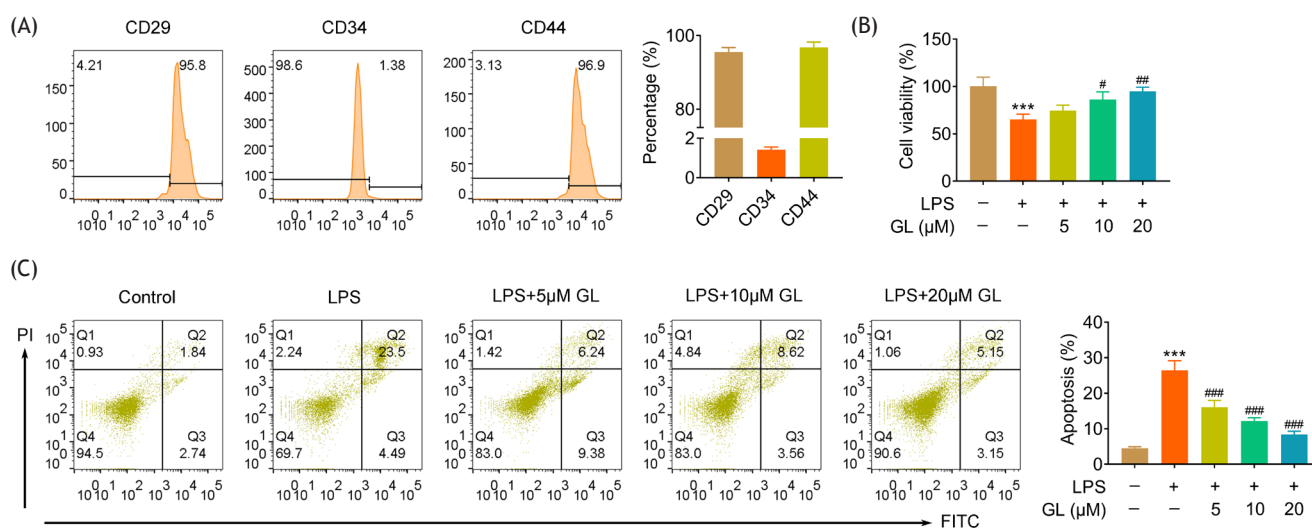


Figure 1 Galangin suppressed cell apoptosis of lipopolysaccharide-treated BMSCs. (A) Flow cytometry showed negative CD34 and positive CD29 and CD44 expression in the isolated BMSCs. (B) Incubation with galangin increased cell viability of lipopolysaccharide-treated BMSCs in a dosage-dependent manner. (C) Incubation with galangin reduced the cell apoptosis of lipopolysaccharide-treated BMSCs in a dosage-dependent manner. # *p* < 0.05, ## *p* < 0.01, ###, *** *p* < 0.001.

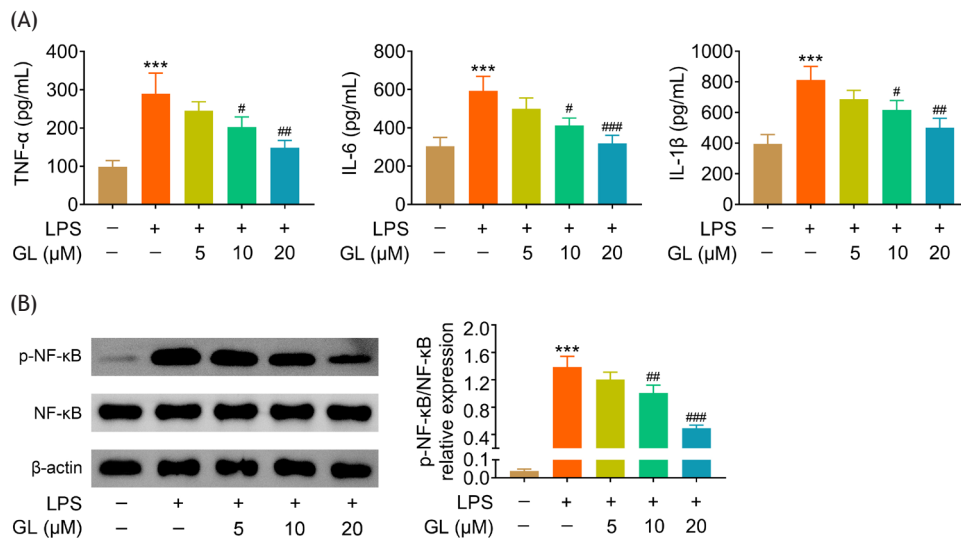


Figure 2 Galangin suppressed lipopolysaccharide-induced inflammation in BMSCs. (A) Incubation with galangin reduced TNF-α, IL-6, and IL-1β levels in lipopolysaccharide-treated BMSCs in a dosage dependent way. (B) Incubation with galangin attenuated lipopolysaccharide-induced increase of p-NF-κB in BMSCs in a dosage-dependent manner. # $p < 0.05$, ## $p < 0.01$, ###, *** $p < 0.001$.

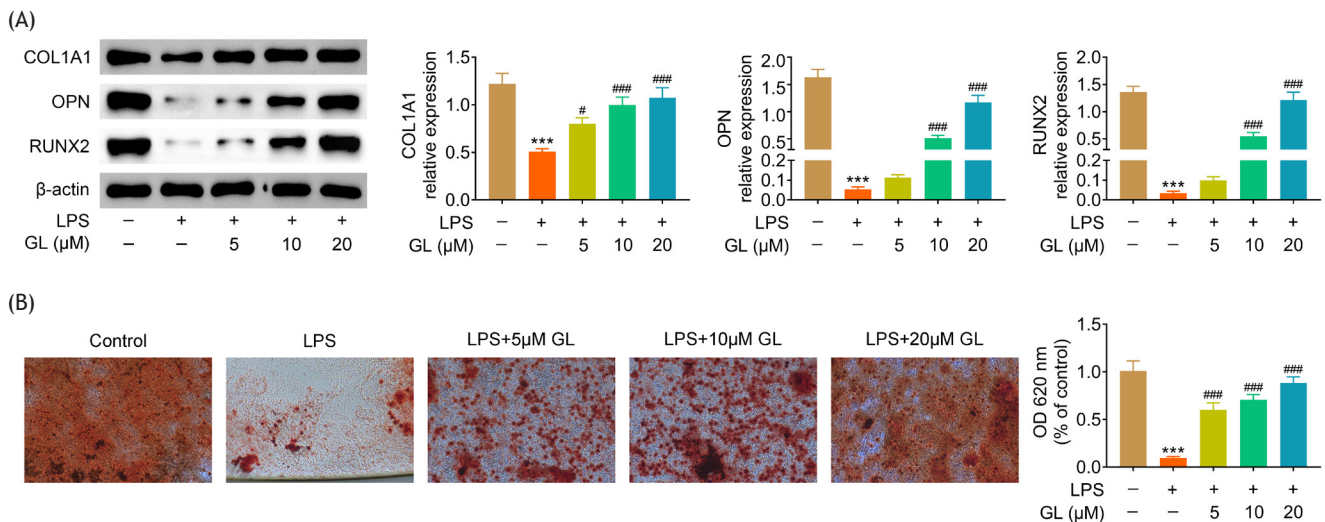


Figure 3 Galangin promoted osteogenic differentiation of lipopolysaccharide-treated BMSCs. (A) Incubation with galangin enhanced COL1A1, OPN, and RUNX2 expression in lipopolysaccharide-treated BMSCs in a dosage-dependent manner. (B) Alizarin Red staining demonstrated that incubation with galangin enhanced the mineralization of lipopolysaccharide-treated BMSCs in a dosage-dependent manner. # $p < 0.05$, ###, *** $p < 0.001$.

protected BMSCs against lipopolysaccharides through promotion of osteogenic differentiation.

Galangin activated AKT/mTOR signaling in lipopolysaccharide-treated BMSCs

Protein expression of AKT and mTOR in BMSCs were not affected by incubation with lipopolysaccharides or galangin. (Figure 4). Phosphorylation of AKT and mTOR in BMSCs were down-regulated by lipopolysaccharides (Figure 4), whereas galangin up-regulated the phosphorylation of AKT and mTOR in lipopolysaccharide-treated BMSCs (Figure 4), indicating that AKT/mTOR signaling in lipopolysaccharide-treated BMSCs was activated by galangin treatment.

Discussion

Traditional Chinese medicines have been shown to play a critical role in bone loss-related diseases.¹⁶ For example, extracts of *Alpinia officinarum* inhibited osteoclast differentiation and bone resorption, while enhanced osteoblast mineralization to prevent pathological bone diseases.¹⁷ Galangin, bioactive flavonoid isolated from *Alpinia officinarum*, suppressed osteoclast formation and inflammation and promoted osteogenic differentiation in various diseases.¹³⁻¹⁵ This study found that galangin exerted anti-inflammatory effect against lipopolysaccharide-treated BMSCs, and stimulated the osteogenic differentiation to prevent immuno-inflammatory oral diseases.

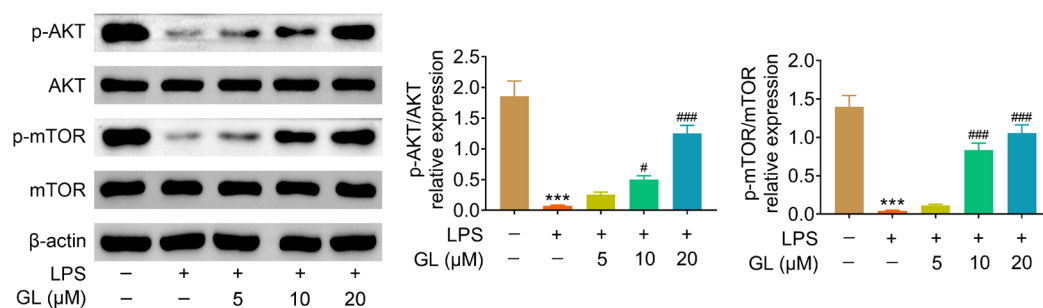


Figure 4 Galangin activated AKT/mTOR signaling in lipopolysaccharide-treated BMSCs. Incubation with galangin enhanced p-AKT and p-mTOR expression in lipopolysaccharide-treated BMSCs in a dosage-dependent manner. # $p < 0.05$, ###, *** $p < 0.001$.

BMSCs, with the multidirectional differentiation potential and self-renewal ability, are significantly related to bone formation and resorption.¹⁸ BMSCs contribute to osseointegration through colonization of osteoblasts into the implant surface, promote alveolar bone regeneration, and facilitate for the treatment of immuno-inflammatory oral diseases.¹⁹ However, the chronic inflammatory micro-environment in immuno-inflammatory oral diseases influences the biological characteristics of stem cells, represses the repair and regeneration abilities.²⁰ Cell viability of BMSCs was down-regulated in lipopolysaccharide-stimulated inflammatory environment, and the osteogenic differentiation was also suppressed.²¹ Therefore, suppressing inflammation of lipopolysaccharide-treated BMSCs might be a promising strategy for the prevention of immuno-inflammatory oral diseases. In this study, BMSCs, with the main characteristics of negative CD34 and positive CD29 and CD44 expression²² were isolated from rat bone marrow. Lipopolysaccharide treatment reduced cell viability of BMSCs, promoted cell apoptosis, and inhibited osteogenic differentiation through the down-regulation of COL1A1, OPN and RUNX2. A previous study reported that galangin promoted alkaline phosphatase activity, and increased expression of osteoblast differentiation markers (osteopontin, osteocalcin, COL1A1, alkaline phosphatase) to induce osteogenic differentiation in osteosarcoma cells.¹⁵ Here, galangin increased cell viability of lipopolysaccharide-treated BMSCs, suppressed the cell apoptosis, and promoted osteogenic differentiation through up-regulation of COL1A1, OPN, and RUNX2 in a dosage-dependent manner. Hence, galangin might be useful for treating immuno-inflammatory oral diseases.

Emerging evidence has shown that lipopolysaccharides induced the up-regulation of TNF- α , IL-1 β , and IL-6 in BMSCs, contributed to the inflammatory responses involved in the pathogenesis of immuno-inflammatory oral diseases.²³ Galangin suppressed lipopolysaccharide-stimulated inflammation through the down-regulation of TNF- α and IL-6.²⁴ Results from this study demonstrated that galangin exerted an anti-inflammatory effect against lipopolysaccharide-treated BMSCs with decreased TNF- α , IL-1 β , and IL-6 levels. NF- κ B signaling, essential for the secretion of proinflammatory factors, was stimulated by lipopolysaccharides.²⁵ The protective effect of galangin against lipopolysaccharide-induced acute inflammation in the lung was associated with NF- κ B signaling inactivation.²⁶ Here, the protein expression of p-NF- κ B in lipopolysaccharide-treated BMSCs was reduced by galangin

treatment in a dosage-dependent manner, suggesting that galangin protected BMSCs against lipopolysaccharide-induced inflammation through NF- κ B signaling inactivation.

PI3K/AKT/mTOR signaling has been shown to be involved in synovial inflammation, subchondral bone dysfunction, and cartilage degradation, thus implicated in the pathogenesis of osteoarthritis.²⁷ Activation of PI3K/AKT/mTOR signaling prevented bone loss,²⁸ and promoted the osteogenic differentiation of BMSCs.²⁹ Galangin promoted the activation of the AKT/mTOR pathway to ameliorate insulin resistance,³⁰ suppress okadaic acid-induced cytotoxicity in neurons.³¹ Results from this study also indicated that galangin attenuated lipopolysaccharide-induced the down-regulation of p-AKT and p-mTOR in BMSCs in a dosage-dependent manner, thus promoting the AKT/mTOR signaling activation.

To sum up, galangin enhanced the cell viability of lipopolysaccharide-treated BMSCs and suppressed cell apoptosis. Moreover, galangin suppressed inflammation and promoted osteogenic differentiation of lipopolysaccharide-treated BMSCs through NF- κ B signaling inactivation and AKT/mTOR signaling activation, respectively. However, the *in vivo* role of galangin in bone loss-related diseases should be investigated further.

Competing Interest

The authors declare no no conflicts of interest.

Ethics Approval

Ethical approval was obtained from the Ethics Committee of The Sixth Affiliated Hospital of Xinjiang Medical University.

Data Availability

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

Author Contributions

Xiaoting Wang and Xinglei Xiao designed and conducted the experiments, analyzed and interpreted the data,

and prepared the manuscript. All authors have read and approved the manuscript.

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