

Geraniin inhibits TNF- α -induced impairments of osteogenesis through NF- κ B and p38 MAPK signalling pathways in bone marrow stem cells

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To cite: Lu C, Gao S, Xu G. Geraniin inhibits TNF- α -induced impairments of osteogenesis through NF- κ B and p38 MAPK signalling pathways in bone marrow stem cells. *Stroke and Vascular Neurology* 2017;**2**: e000046. doi:10.1136/svn-2016-000046

Received 13 October 2016
Revised 15 February 2017
Accepted 16 February 2017
Published Online First
21 April 2017

ABSTRACT

Objective To study the effect and mechanism of geraniin on the osteogenesis of tumour necrosis factor- α (TNF- α)-induced bone marrow-derived mesenchymal stem cells (BMSCs).

Methods BALB/c mice aged 4–6 weeks were used to collect BMSC. Methyl thiazol tetrazolium assay and lactate dehydrogenase assay were used to analyse the effect of geraniin on the TNF- α -induced impairments of osteogenesis in BMSCs. BMSCs were stained using Oil red to measure the osteogenesis. Real-time reverse transcription PCR and western blot analysis were used to analyse the expression of RunX2 and Osx miRNA, and expression of NF- κ B, I κ B- α and p38 MAPK protein in BMSCs.

Results 2.5 μ M geraniin significantly inhibited TNF- α -induced BMSCs cytotoxicity. 2.5 μ M geraniin significantly reduced the expression of RunX2 and Osx miRNA in TNF- α -induced BMSCs. 2.5 μ M geraniin suppressed the expression of NF- κ B and p38 MAPK protein and promoted the expression of I κ B- α protein in the TNF- α -induced BMSCs.

Conclusion Geraniin inhibits TNF- α -induced impairments of osteogenesis through NF- κ B/I κ B- α and p38 MAPK signalling pathways in BMSCs.

INTRODUCTION

The normal bone metabolism is extremely essential for maintaining the bone mineral density and bone strength, which is mainly determined by a delicate balance between bone formation by anabolic activities of osteoblasts and bone resorption by catabolic effects of osteoclasts.¹ Since bone marrow is a good source of mesenchymal stem cells which have clonogenicity and multipotential characteristics, it is the most commonly used cell therapy for bone regeneration in the orthopaedics.² Bone marrow-derived mesenchymal stem cells (BMSCs), a kind of adult stem cells, have the same hereditary basis with other tissues.³ BMSCs can be differentiated into osteoblasts, chondrocytes, adipocytes and myoblasts.⁴ Mankani *et al* reported that transplantation of BMSC-conjugated hydroxyapatite/tricalcium

phosphate particles was able to heal craniofacial bone defects for a long term.⁵ Quarto *et al* first presented the preliminary clinical data of three patients with the treatment of autologous BMSCs and hydroxyapatite porous blocks in long bone defects. Complete fusion was observed in all three patients.⁶ Therefore, the proliferation and differentiation of BMSCs is critical for the osteogenesis.⁷

Increasing evidences proved that the inflammation could disturb the balance between bone formation and resorption resulting in osteoporosis or osteonecrosis.⁸ The proliferation and osteogenic differentiation of BMSCs could be also affected by inflammatory cytokines including tumour necrosis factor- α (TNF- α), interleukin 1 (IL-1), IL-6 and interferon γ (IFN- γ).⁹ TNF- α is an important inflammatory cytokine which can regulate cellular proliferation, differentiation and apoptosis by activating various signal pathways and molecular events.¹⁰ TNF- α can cause osteoclast-induced bone destruction, inhibit the osteoblastogenesis and regulate the migration of BMSCs in vivo.^{11 12} Lu *et al* demonstrated that TNF- α inhibited osteoblasts differentiation from precursor cells, reduced expression of RUNX2 and osteoblast-associated transcription factors (Osterix) and suppressed vitamin D-stimulated transcription owing to the activation of transcription factor Nuclear factor kappa-light-chain-enhancer of activated B cell (NF- κ B).¹³ Hess *et al* showed that TNF- α increased the expression of bone morphogenetic protein-2 (BMP-2) in BMSCs through NF- κ B signalling pathway in early osteogenic differentiation and NF- κ B stimulated key regulators of osteogenesis, such as BMP-2, RUNX2 and Osterix, resulting in enhanced mineralisation of the extracellular matrix.¹⁴ Huang *et al* reported that levels of Runx2, Osx and ALP were upregulated at lower concentration of TNF- α , but downregulated at higher



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concentration of TNF- α causing dose-dependent effects of TNF- α on BMSCs' osteogenic differentiation.¹⁵ These findings suggested that the binding of TNF- α to its receptors resulted in activating multiple signalling pathways.

Geraniin is a kind of polyphenols extracted from under leaf pearl, which is widely used to treat hepatitis, enteritis, dysentery, nephritis and oedema.¹⁶ Recently, Okabe *et al* reported that geraniin could relieve pain and reduce blood pressure through inhibiting releasing TNF- α .¹⁷ Xiao *et al* demonstrated that geraniin inhibited RANKL-induced osteoclastogenesis in a dose-dependent manner through NF- κ B and Extracellular-signal Regulated protein Kinase (ERK) signalling pathways, which might be one of the mechanisms of anti-osteoporosis effects.¹⁸

In this study, the effects of geraniin on TNF- α -induced impairments of osteogenesis in BMSCs were studied by using methyl thiazol tetrazolium (MTT) assay and lactate dehydrogenase (LDH) assay. Oil red was used to measure osteogenesis of BMSCs. Real-time PCR and western blot analysis were used to analyse the miRNA expression of RunX2 and *Osx*, and detect the protein expression of NF- κ B/Inhibitor of nuclear factor kappa-B, alpha ($\text{I}\kappa\text{B}-\alpha$) and p38 Mitogen Activated Proteinkinase (MAPK). In summary, the aim of this study is to unveil the mechanisms of the inhibitory effects of geraniin on TNF- α -induced impairments of osteogenesis in BMSCs.

MATERIALS AND METHODS

Isolation and culture of BMSCs

All experiments were performed following the guidelines of Animal Use and Care Committee. BALB/c mice aged 4–6 weeks were aseptically raised in the clean animal room. The mice BMSCs were harvested from long bones in the aseptic condition after carefully trimming excess tissues and washing marrow cavity using Phosphate Buffered Solution at 4°C. Subsequently, BMSCs were centrifugated at 2000 rpm for 5 min prior to resuspending with 5 mL Dulbecco's modified eagle medium (Gibco, USA) plus 10% fetal bovine serum (Gibco, USA) in 25 cm² flasks. After 24 hours, the floating cells were removed and the adherent cells were cultured at 37°C with 5% humidified CO₂.

The adherent cells were passaged by trypsinisation when reaching 80%–90% confluency and subcultured at a density of 4000–5000 cells/cm². Meanwhile, adherent cells were incubated with 0.25% trypsin and 1 mM Ethylene Diamine Tetraacetic Acid at 37°C for 5 min. BMSC were subcultured four times and incubated for 3 days after labelling with 3 μ g/mL of bromodeoxyuridine.¹⁹

MTT assay

BMSCs were seeded into a 96-well plate at a density of 2 \times 10⁴ cells/well, and the fresh medium was supplemented with 20 ng/mL TNF- α or 0, 1.25, 2.5, 5, 10, 20 μ M geraniin for 5 days. Subsequently, 20 μ L 5 mg/mL MTT (Sigma) was added into each well. After 4-hour incubation, the absorbancy of each well was measured by using

an ELX800 absorbance microplate reader (Bio-Tek, USA) at 560 nm.

LDH assay

BMSCs were seeded into a 96-well plate at a density of 2 \times 10⁴ cells/well, and the fresh medium was supplemented with 20 ng/mL TNF- α or 2.5 μ M geraniin for 1 day. Then, 50 μ L LDH was added into each well at 37°C for 30 min protecting from light. The absorbancy was measured at 450 nm using an ELX800 absorbance microplate reader.

Oil red O stain

BMSCs were seeded into a 96-well plate at a density of 2 \times 10⁶ cells/well, and the fresh medium was supplemented with 20 ng/mL TNF- α or 2.5 μ M geraniin for 1 day. BMSCs was fixed by using 5% precool paraformaldehyde for 30 min at 4°C. 0.6% (w/v) oil red was added into each well and stained for 15 min at 37°C. BMSCs were carefully washed to remove unbound dyes and then immersed with 1 mL isopropyl alcohol for 10 min at 37°C. BMSCs were observed through fluorescent microscope (D5300, Nikon, Japan) at 510 nm. Three different visual fields of control group and geraniin-treated group containing approximately 10 cells were analysed, and triplicate experiments were done at three independent time points. The mean fluorescence intensity (MFI) of the whole cell was separately analysed by Image J software.

Real-time reverse transcription PCR

BMSCs were seeded into a 96-well plate at a density of 2 \times 10⁶ cells/well and the fresh medium was supplemented with 20 ng/mL TNF- α or 2.5 μ M geraniin for 1 day. RNeasy Mini kit (Qiagen, USA) was used to collect total RNA from each well according to the manufacturer's instructions. One microgram of total RNA was used to synthesise cDNA using reverse transcriptase (TaKaRa Biotechnology, Japan). Then, the expression of miRNA was detected by using Real-time PCR ABI 7500 Sequencing Detection System (Applied Biosystems, USA) and a SYBR Premix Ex Tag kit (TaKaRa Biotechnology, Japan). The primers of Runx2 were as follows: 5'-TCTTAGAACAAATTCTGCCCTTT-3' and 5'-TGCTTTGGTCTTGAAATCACA-3'; the primers of *Osx* were as follows: 5'-CCTCCTCAGCTCACCTTCTC-3' and 5'-GTTGGGAGCCCAAATAGAAA-3'; the primers of GAPDH were as follows: 5'-GAAGGTGAAGTC-GGAGTC-3' and 5'-GAGATGGTGATGGGATTTC-3'. Real-time PCR was performed under the following conditions: 40 cycles of 95°C for 35 s and 60°C for 35 s.²⁰

Western blot analysis

BMSCs were seeded into a 96-well plate at a density of 2 \times 10⁶ cells/well, and the fresh medium was supplemented with 20 ng/mL TNF- α or 2.5 μ M geraniin for 1 day. BMSCs were lysed using radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific, USA) for 20–30 min on ice. The protein concentration was measured using a bicinchoninic acid protein assay (Thermo Fisher Scientific, USA). Thirty microgram proteins were

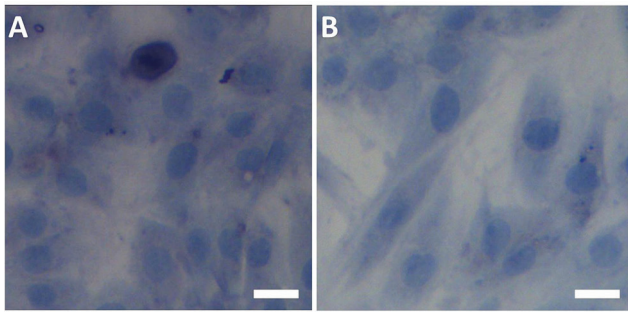


Figure 1 The morphology of BMSCs was observed by light microscope. (A) Primary BMSCs cultured for 24 hours; (B) primary BMSCs cultured for 4 days. The scale bar is 10 μ m. BMSCs, bone marrow-derived mesenchymal stem cells.

separated using 10%–12% SDS-PAGE (Beyotime Institute of Biotechnology, China), transferred onto polyvinylidene difluoride membranes, blocked with 5% skimmed milk in TBS–Tween (0.2% Tween-20) for 2 hour at 37°C and then incubated with primary antibodies overnight at 4°C including anti-NF- κ B/p65 (1:2000, Cell Signaling Technology, USA), anti-I κ B- α (1:2000, Cell Signaling Technology, USA), anti-p38 MAPK(1:2000, Cell Signaling Technology, USA) and β -actin (Beyotime Institute of Biotechnology, China). Membranes were incubated with the appropriate secondary antibodies (Beyotime Institute of Biotechnology, China) conjugated with an IRDye 800CW. Immunoblots were analysed with Image-Pro Plus 5.0 software (Media Cybernetics, USA).

Statistical analysis

All data in graphs are generated from at least three independent experiments and expressed as the mean \pm SD. Student's *t*-test was used to determine the statistical significance, and values of $p < 0.05$ were considered to be statistically significant.

RESULTS

Identification of BMSCs

Cell morphology was observed by a light microscope with staining of bromodeoxyuridine. After 24 hours of incubation, most BMSCs had already adhered to the surface of culture flasks (figure 1A). BMSCs exhibited a fibrocyte-like

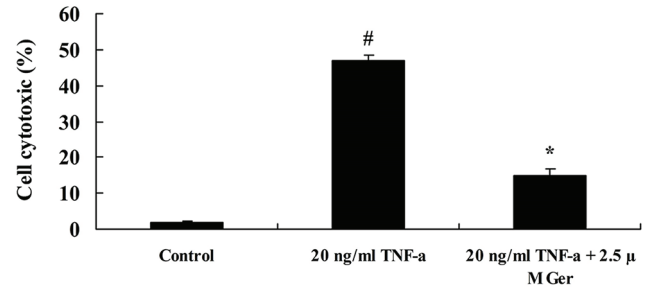


Figure 3 Effect of geraniin on TNF- α -induced BMSCs cytotoxicity. # $p < 0.01$ compared with control group, * $p < 0.05$ compared with 20 ng/mL TNF- α group. BMSCs, bone marrow-derived mesenchymal stem cells; Ger, geraniin; TNF- α , tumour necrosis factor-alpha.

form with the blue nucleus and a long spindle shape after being cultured for 4 days (figure 1B).

Effect of geraniin on TNF- α -induced BMSCs cytotoxicity

MTT assay was used to detect the effect of geraniin on TNF- α -induced BMSCs' viability. After being treated with 2.5 μ M geraniin for 1 day, the viability of BMSCs was significantly increased, compared with control group ($p < 0.01$). However, the viability of BMSCs was significantly suppressed with treatment of 20 μ M geraniin at 3 days ($p < 0.01$). After being treated with 10 or 20 μ M geraniin for 5 days, the BMSCs' viability significantly decreased ($p < 0.01$) (figure 2A). Therefore, treating with 2.5 μ M geraniin for 1 day was selected as optimal condition in this study. The viability of BMSCs was significantly reduced after treating with 20 ng/mL TNF- α ($p < 0.01$); however, 2.5 μ M geraniin could significantly improve TNF- α -induced BMSCs cytotoxicity ($p < 0.05$) (figure 2B). Moreover, LDH assay was used to investigate the effect of geraniin on TNF- α -induced BMSCs cytotoxicity. As shown in figure 3, TNF- α showed significant BMSCs cytotoxicity at the dose of 20 ng/mL. In contrast, 2.5 μ M geraniin could significantly diminished TNF- α -induced BMSCs cytotoxicity (figure 3).

Effect of geraniin on osteogenesis in BMSCs

To investigate the effect of geraniin on osteogenesis in BMSCs, Oil red O stain was used to detected osteogenesis of BMSCs. As shown in figure 4, the red area of control group (figure 4A) was lower than that of geraniin group

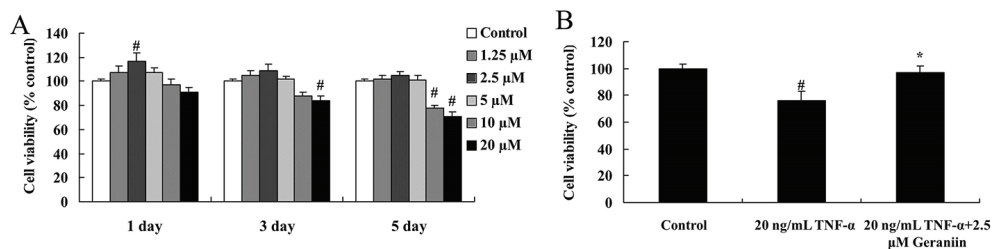


Figure 2 (A) The effects of geraniin with different concentrations on BMSCs' viability; (B) effect of geraniin on TNF- α -induced BMSC viability. # $p < 0.01$ compared with control group, * $p < 0.05$ compared with 20 ng/mL TNF- α group. BMSCs, bone marrow-derived mesenchymal stem cells; TNF- α , tumour necrosis factor-alpha.

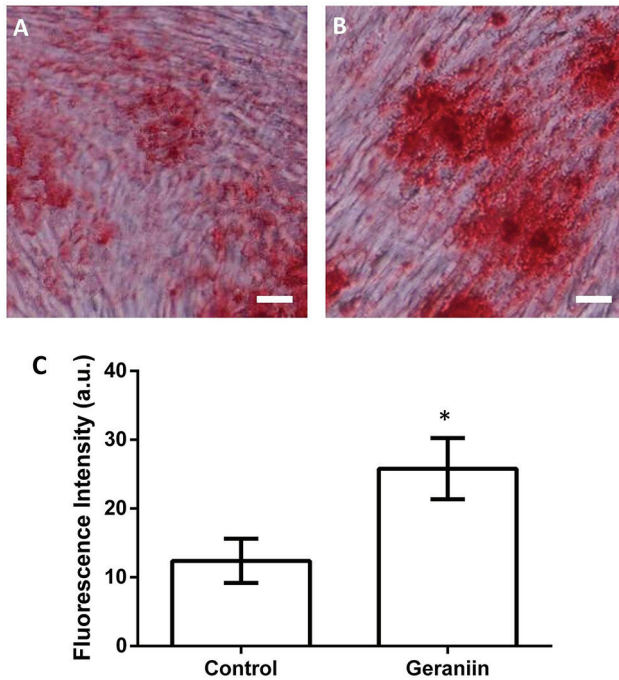


Figure 4 Effect of geraniin on osteogenesis in BMSCs. * $p < 0.01$ compared with control group; the scale bar is 10 μm . BMSCs, bone marrow-derived mesenchymal stem cells.

(figure 4B). The MFI of BMSCs nucleus in the control group was significantly lower than that of geraniin group ($p < 0.05$) (figure 4C).

The expression of miRNA Runx2 and Osx

To unveil the mechanisms on the effect of geraniin on TNF- α -induced impairment of BMSCs, the expression of miRNA Runx2 and Osx were measured using real-time PCR in this study. The expression of miRNA Runx2 and Osx was significantly decreased in BMSCs after treating with 20 ng/mL TNF- α compared with control group. In contrast, the expression of miRNA Runx2 and Osx was significantly upregulated by treating with 2.5 μM geraniin (figure 5).

Effect of geraniin on TNF- α -activated signal pathways in BMSCs

To investigate mechanisms on the effect of geraniin on TNF- α -induced impairment of BMSCs, NF- κB /p65, p-I κB - α and p38 MAPK protein expression was detected using western blot analysis. As shown in figure 6A, the expression of NF- κB /p65 and p38 MAPK protein in TNF- α -treated BMSCs was higher than that in control group; however, TNF- α significantly inhibited the expression of p-I κB - α protein in BMSCs. After treating with 2.5 μM geraniin, the expression of NF- κB /p65 and p38 MAPK significantly reduced in BMSCs. In contrast, geraniin

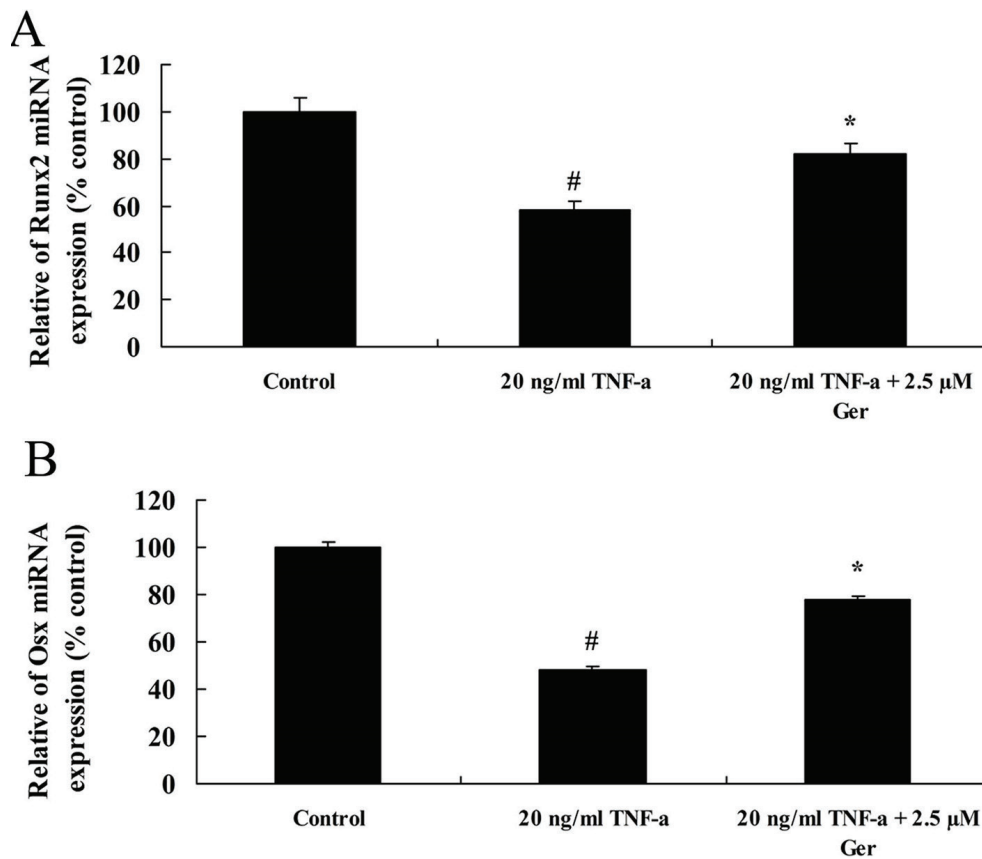


Figure 5 The expression of Runx2 (A) and Osx (B) in BMSCs treating with TNF- α or TNF- α +Ger. # $p < 0.05$ compared with control group, * $p < 0.05$ compared with TNF- α group. BMSCs, bone marrow-derived mesenchymal stem cells; Ger, geraniin; TNF- α , tumour necrosis factor-alpha.

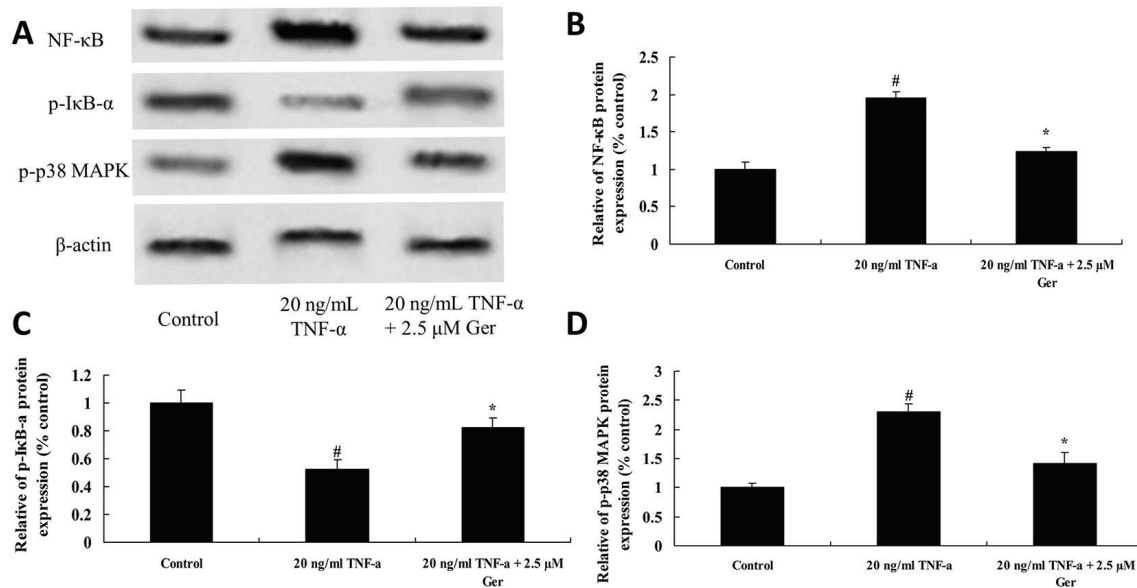


Figure 6 Effect of geraniin on TNF- α -activated NF- κ B, p-I κ B- α and p38 MAPK protein expression in BMSCs, including western blot analysis (A) and statistics of NF- κ B (B), p-I κ B- α (C) and p38 MAPK (D) protein expression. # $p < 0.01$ compared with control group, * $p < 0.05$ compared with TNF- α group. BMSCs, bone marrow-derived mesenchymal stem cells; Ger, geraniin; TNF- α , tumour necrosis factor-alpha.

significantly increased the expression of p-I κ B- α protein in TNF- α -treated BMSCs (figure 6B-D).

DISCUSSION

BMSCs are pluripotent stem cells derived from various organs or tissues. In the normal conditions, most of BMSCs stay in the dormant state; however, under pathological conditions, the renewability of BMSCs will be activated.²¹ BMSCs can be differentiated into osteoblasts, chondrocytes, adipocytes and myoblasts.⁴ Various evidences demonstrated that BMSCs played an essential role in osteogenesis and inflammatory cytokines could disrupt the balance between bone formation and bone resorption.^{1 2} TNF- α is an important inflammatory cytokine regulating proliferation and differentiation of BMSCs, resulting in osteoclast-induced bone destruction and inhibiting osteogenesis.¹⁰⁻¹² In this study, we demonstrated that 2.5 μ M geraniin could decrease TNF- α -induced BMSCs cytotoxicity. Lu *et al* indicated that geraniin was able to prevent ovariectomy-induced osteoporosis in rats.¹⁶ Park *et al* suggested that soybean-treated osteoblasts suppressed TNF- α -stimulated osteoclast formation and RANKL-induced osteoclast differentiation through increasing OPG/RANKL ratio.²² Previous study showed that geraniin played a role of TNF- α inhibitor on impairments of osteogenesis.¹⁸ In this study, we found that geraniin could significantly improve expression of Runx2 and Osx miRNA in BMSCs. Velázquez-González *et al* also showed that geraniin possessed antinociceptive and anti-inflammatory activities.²³ As we all know, Runx2 is the most important osteogenic transcriptional factor playing a determinant role in the early stage of osteogenesis and is a significant marker of early osteogenic differentiation.²⁴ It has been proved that TNF- α can stimulate osteoclast

formation via NF- κ B pathway.²⁵ Recently, Fujii *et al* reported that TNF- α promoted the differentiation of osteogenesis through activation of NF- κ B pathways.²⁶ In this study, geraniin significantly suppressed TNF- α -induced expression of NF- κ B/p65 protein and promoted p-I κ B- α protein expression in BMSCs. Similarly, Xiao *et al* suggested that geraniin suppressed RANKL-induced NF- κ B expression.¹⁸ Moreover, we suggested that geraniin could significantly reduce the TNF- α -induced p38 MAPK protein expression in BMSCs. Adhesion molecules are produced through stimulation of BMSCs by p38 MAPK signalling pathways.²⁷ TNF- α can promote the secretion of Hepatocyte growth factor by BMSCs through p38 MAPK and PI3K/Akt pathways.²⁸ Thus, NF- κ B, ERK and c-Jun-NH2-terminal Kinase (JNK) pathways play an essential role for migration homing and adhesion of BMSCs.²⁹

In summary, geraniin can inhibit TNF- α -induced impairments in BMSCs. Moreover, the mechanisms about protective effect of geraniin are mediated through NF- κ B, I κ B- α and p38 MAPK signalling pathways in BMSCs. It suggests that geraniin may be a new candidate drug for treating TNF- α -induced impairment of osteogenesis.

Contributors CL: guarantor of integrity of the entire study, study concepts and manuscript review; CL and SG: study design, statistical analysis and manuscript editing; GX: definition of intellectual content; SG and GX: experimental studies, data acquisition, data analysis and manuscript preparation.

Competing interests None declared.

Provenance and peer review Not commissioned; externally peer reviewed.

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