

Original Paper

# Quercetin Triggers Apoptosis of Lipopolysaccharide (LPS)-induced Osteoclasts and Inhibits Bone Resorption in RAW264.7 Cells

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## Key Words

Quercetin • Osteoclast • LPS • Receptor activator of nuclear factor- $\kappa$ B (RANK) • Cyclooxygenase-2 (COX-2) • Mitogen-activated protein kinases (MAPK)

## Abstract

**Aims:** Quercetin, a flavonoid present in vegetables, has anti-inflammatory properties and potential inhibitory effects on bone resorption. Up to date, the effect of quercetin on lipopolysaccharide (LPS)-induced osteoclastogenesis has not yet been reported. In the current study, we evaluated the effect of quercetin on LPS-induced osteoclast apoptosis and bone resorption. **Methods:** RAW264.7 cells were non-treated, treated with LPS alone, or treated with both LPS and quercetin. After treatment, the number of osteoclasts, cell viability, bone resorption and osteoclast apoptosis were measured. The expressions of osteoclast-related genes including tartrate-resistant acid phosphatase (*TRAP*), matrix metalloproteinase-9 (*MMP-9*) and cathepsin K (*CK*) were determined by real-time quantitative polymerase chain reaction (qPCR). Protein levels of receptor activator of nuclear factor- $\kappa$ B (RANK), tumor necrosis factor receptor-associated factor 6 (TRAF6), cyclooxygenase-2 (COX-2), Bax, Bcl-2 and mitogen-activated protein kinases (MAPKs) were measured using Western blotting assays. The MAPK signaling pathway was blocked by pretreatment with MAPK inhibitors. **Results:** LPS directly promoted osteoclast differentiation of RAW264.7 cells and upregulated the protein expression of RANK, TRAF6 and COX-2; while quercetin significantly decreased the number of LPS-induced osteoclasts in a dose-dependent manner. None of the treatments increased cytotoxicity in RAW264.7 cells. Quercetin inhibited mRNA expressions of osteoclast-related genes and protein levels of RANK, TRAF6 and COX-2 in LPS-induced mature osteoclasts. Quercetin also induced apoptosis and inhibited bone resorptive activity in LPS-induced mature osteoclasts. Furthermore, quercetin promoted the apoptotic signaling pathway including increasing the phosphorylation of p38-MAPK, c-Jun N-terminal kinases/stress-activated protein kinases

(JNK/SAPK), and Bax, while inhibited Bcl-2 expression. **Conclusions:** Quercetin could suppress LPS-induced osteoclast bone resorption through blocking RANK signaling and inhibiting the expression of osteoclast-related genes. Quercetin also promoted LPS-induced osteoclast apoptosis via activation of the MAPK apoptotic signaling pathway. These findings suggest that quercetin could be of potential use as a therapeutic agent to treat bacteria-induced bone resorption.

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## Introduction

Bone is a dynamic tissue that constantly undergoes remodeling where a coupled process of bone formation and resorption continues throughout life [1]. This process allows physiological bone growth, repair of damaged bones, and is important for the regulation of systemic calcium and phosphate levels. Chronic osteomyelitis, a bone infectious disease, is a dysregulation of this process, which results in excessive bone resorption. The receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) is a key factor in regulating the process of osteoclast differentiation and maintaining the survival of mature osteoclasts [2]. RANK signaling plays a critical role in regulating osteoclast differentiation and bone resorptive activity. RANK is activated by RANKL via the tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), which in turn induces the activation of mitogen-activated protein (MAP) kinases (MAPK) [3].

Lipopolysaccharide (LPS), a component of the outer membranes of all Gram-negative bacteria, is the first bacterial component shown to be capable of inducing bone resorption *in vitro* [4]. LPS induces production of cytokines including TNF- $\alpha$  by fibroblasts, macrophages, and other cells, which can further induce osteoclast formation and activation [5]. Moreover, LPS can promote osteoclast differentiation, fusion, survival and activation independent of IL-1, TNF- $\alpha$ , and RANKL [6-9]. However, up to date, effective therapeutic treatments for LPS-induced bone destruction are limited to antibiotics and surgical repairs in chronic inflammatory diseases. Therefore, the research and development of potential drugs that can inhibit LPS induced osteolysis remains a major goal in the prevention of bone destruction in infective bone diseases.

Quercetin, as a dietary flavonoid, has been highlighted as a bioactive substance due to its biological, pharmacological, and medicinal features. Evidence suggests that quercetin inhibits bone loss by affecting osteoclastogenesis and regulating a variety of systemic and local factors such as hormones and inflammatory cytokines [10-12]. Moreover, quercetin is reported to inhibit LPS-induced MAPK activation, and cyclooxygenase-2 (COX-2) expression [13].

The aim of the current study was to evaluate the effect of quercetin on LPS-induced osteoclast bone resorptive activity and induction of apoptosis. We also aimed to clarify the inhibitory mechanism of quercetin on MAPK phosphorylation, as well as on the expression of RANK/TRAF6 and COX-2 in LPS-induced osteoclasts.

## Materials and Methods

### Reagents

*Escherichia coli* LPS (serotype 055:B5), quercetin, Leukocyte Acid Phosphatase Kit 387-A, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and Hoechst 33258 were purchased from Sigma Co. (St Louis, MO, USA). Protease inhibitor cocktail and selective MAPK inhibitors PD98059, SB203580 and SP600125 were purchased from Calbiochem (San Diego, CA, USA). Alpha modified-minimum essential medium ( $\alpha$ -MEM), 10% fetal bovine serum (FBS), penicillin/streptomycin and TRIZOL<sup>®</sup> reagent were purchased from Gibco (Gibco, Rockville, MD). Primary antibodies against phosphorylated p38-MAPK, total c-Jun N-terminal kinase (JNK), phosphorylated JNK, extracellular signal regulated kinase (ERK1/2),

phosphorylated ERK 1/2, Bax, Bcl-2 and COX-2 were purchased from Cell Signaling Technology (Beverly, MA). Primary antibodies against RANK, TRAF6 and  $\beta$ -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse macrophage RAW 264.7 cell line that can be induced to differentiate into osteoclasts was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Other chemicals and reagents used in this study were of analytical grade.

#### *Cell culture*

RAW 264.7 cells were grown in  $\alpha$ -MEM supplemented with 10% (v/v) FBS, 1% (v/v) penicillin-streptomycin solution, 10 mM HEPES solution and incubated at 37°C in 5% CO<sub>2</sub> humidified air. Medium was changed every 3 d. For the cell viability assay, cells were seeded at  $1 \times 10^3$  cells/cm<sup>2</sup>; for other experiments, cells were seeded at  $4 \times 10^4$  cells/cm<sup>2</sup>. RAW264.7 cells were seeded into culture dishes and incubated overnight before treatment.

To evaluate the effect of LPS-induced osteoclastogenesis, cells were treated with 10, 50, 100, 200, or 1000 ng/mL LPS or without LPS for 4 d until differentiation. To evaluate the effect of quercetin on LPS-induced mature osteoclast apoptosis, RAW264.7 cells were treated with 100 ng/mL LPS for 4 d. On differentiation day 4, cells were treated with 5, 10, 15, 25, or 50  $\mu$ M quercetin or without quercetin in the presence of LPS (100 ng/mL).

#### *Tartrate-resistant acid phosphatase staining*

RAW264.7 cells were incubated with LPS at 100 ng/mL for 4 d. On differentiation day 4, cells were treated with 5, 10, 15, 25, or 50  $\mu$ M quercetin or without quercetin for an additional 24 and 48h in the presence of LPS (100 ng/mL). Cells were washed with phosphate-buffered saline (PBS) and fixed in 4% (v/v) paraformaldehyde at room temperature for 15 min. Cells were stained with TRAP using the Leukocyte Acid Phosphatase Kit 387-A at 37°C for 60 min. Stained cells were then applied for microscopy (Olympus IX71, Olympus Optical, Tokyo, Japan). Osteoclasts were identified as TRAP-positive cells with three or more nuclei in each cell.

#### *Cell viability assay*

Cell viability was measured with the Cell Counting Kit-8 (Dojindo Molecular Technologies, MD) according to the manufacturer's protocol. RAW264.7 cells ( $1 \times 10^3$  cells/cm<sup>2</sup>) were seeded into 96-well flat bottom plates and incubated overnight before experiment. To evaluate the effect of quercetin on pre-osteoclastic cell viability, cells were treated with 5, 10, 15, 25, or 50  $\mu$ M quercetin or without quercetin for 24 h and 4 d. To evaluate the effect of quercetin on the viability of mature osteoclasts, RAW264.7 cells were incubated with LPS at 100 ng/mL for 4 d before treatment with 5, 10, 15, 25, or 50  $\mu$ M quercetin or without quercetin in the presence of LPS (100 ng/mL) for 24 h. The absorbance of each sample was measured at 450 nm using a microplate reader (TECAN, Salzburg, Austria). All treatments were performed in triplicate.

#### *Bone resorption pit assays*

Bone resorption pit assays were conducted on as previously described [14]. RAW264.7 cells ( $1 \times 10^4$  cells/cm<sup>2</sup>) were plated onto 150- $\mu$ m-thick bovine cortical bone slices in culture dishes of 25-mm-diameter and incubated overnight before experiment. The cells were then treated with LPS for 6 d. From differentiation day 6 when the mature osteoclasts were functionally activated, cells were treated with 5, 10, 15, 25, or 50  $\mu$ M quercetin or without quercetin in the presence of 100 ng/mL LPS. Cell culture media was changed every 3 d with the same additions of quercetin and LPS until differentiation day 15, when cells were washed with PBS and fixed in 4% (v/v) paraformaldehyde. Fixed cells were then treated with 2 M NaOH and sonicated to remove cells. The resorption pits were stained with 1% toluidine blue-1% borate for 5 min. The images of resorption pit were visualized and captured with a light microscope (Olympus IX71) and quantified by the number of resorption pits formed per unit area.

#### *Study of osteoclast apoptosis*

To measure apoptosis, we performed Hoechst 33258 staining to visualize nuclear morphology and nucleosomal DNA fragmentation in osteoclasts. RAW264.7 cells ( $4 \times 10^4$  cells/cm<sup>2</sup>) were seeded into 12-well plates and incubated overnight before experiment. The cells were treated with 100 ng/mL LPS for 4 d. On day 4, cells were treated with or without quercetin in the presence of 100 ng/mL LPS for 24h. At the end of the treatment with quercetin, cells were washed to remove non-adherent cells, and the adherent cells were

**Table 1.** Sequences of PCR primers used for real time quantitative PCR.

Gene	Accession No	Sequences(5' → 3')	PCR product size (bp)
TRAP	NM_007388	CACTCCACCCCTGAGATTGT(F)	118
		CATCGTCTGCACGGTCTCTG(R)	
MMP-9	NM_013599	CTGGACAGCCAGACACTAAAG(F)	145
		CTCGCGGCAAGTCTTCAGAG(R)	
Cathepsin K	NM_007802	GAAGAAGACTACCAGAAGCAG(F)	102
		TCCAGGTTATGGGCAGAGATT(R)	
β-actin	NM_007393	GGCTGTATTCCCTCCATCG(F)	154
		CCAGTTGGTAACAATGCCATGT(R)	

fixed in 4% paraformaldehyde solution for 10 min. After wash with PBS, cells were incubated with 0.2 mM Hoechst 33258 for 10 min in the dark. Cells with nuclei containing clearly condensed chromatin or cells with fragmented nuclei were scored as apoptotic; the results were expressed as the number of apoptotic cells. The images of apoptotic osteoclasts were obtained under a fluorescence microscope (Olympus IX71).

#### Real-time QPCR

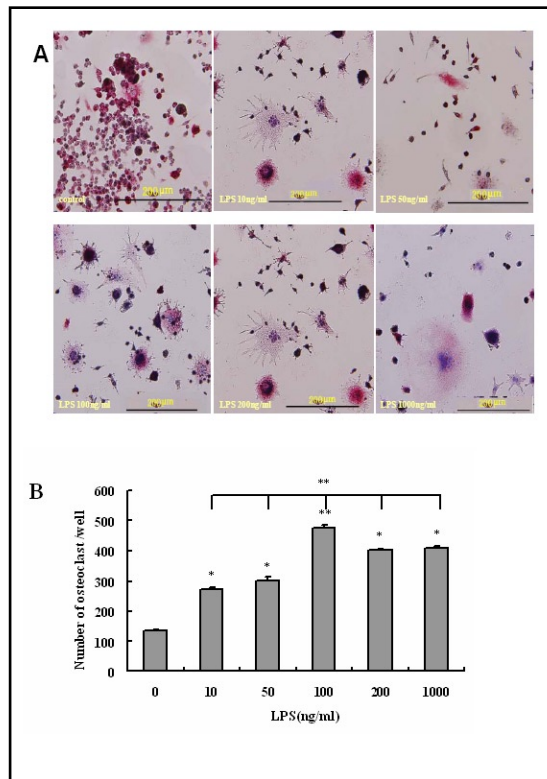
Messenger RNA expression of osteoclast-specific genes including *TRAP*, matrix metalloproteinase-9 (*MMP-9*), and cathepsin K (*CK*) were determined by real-time qPCR. Total RNA was extracted from RAW264.7 cells either treated with 10, 50, 100, 200, or 1000 ng/mL LPS at day 4, or treated with LPS at 100 ng/mL for 4 d, then with 5, 10, 15, 25, or 50 μM quercetin in the presence of 100 ng/mL LPS for 24 h. Total RNA was isolated using Trizol reagent and quantified by spectrophotometry. After isolation, 2 μg total RNA from each sample was used for cDNA synthesis using the M-MLV1<sup>st</sup> Strand Kit (Invitrogen) according to the manufacturer's protocol. Primers for *TRAP*, *MMP-9*, cathepsin K, and β-actin are presented in Table 1. All real-time qPCR reactions were performed in the ABI PRISM 7700 sequence detection system (Applied Biosystems, Hamilton, New Zealand). In each reaction, 1 μL cDNA, 9 μL 2.5×SYBR Green RealMasterMix (Tiangen Biotech, Beijing, China), and 0.25 μM forward and reverse primer in a total volume of 20 μL were used. The reaction condition was as follows: 1 cycle of 95°C for 2 min followed by 40 cycles of 95°C for 20 sec, 63°C for 20 sec, and 68°C for 30 sec. Real-time qPCR for each sample was run in triplicate. β-actin was used as internal control, and all results were analyzed using the standard 2<sup>-ΔΔCT</sup> method described previously [15]. A Student's t-test was conducted to determine significant differences ( $P < 0.05$ ) between two treatment groups.

#### Western blot analysis

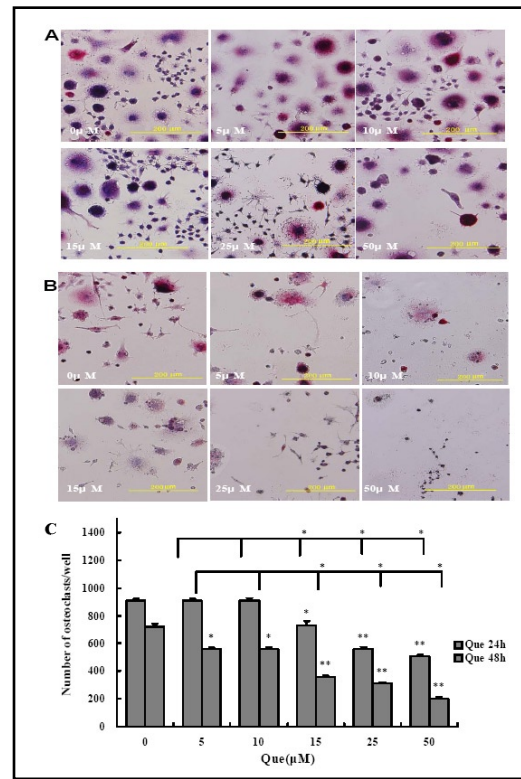
At the end of treatment, cell culture medium was aspirated and cells were detached in PBS by scrapping. Detached cells were transferred to fresh microcentrifuge tubes and centrifuged at 5000 rpm at 4°C for 10 min. Cell pellets were then lysed in 300 μL lysis buffer (Cytobuster protein extraction reagent, Novagen, Darmstadt, Germany) with 25 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1×protease inhibitor cocktail. Protein concentrations were determined by standard Bradford assay. For Western blotting, equal amount of protein from each sample was loaded on SDS-PAGE and electrotransferred onto PVDF membranes (Millipore, Bedford, MA, USA). These membranes were then blocked with 5% (w/v) bovine serum albumin in TBST [10 mM Tris, 150 mM NaCl, and 0.1% (v/v) Tween 20, pH = 7.5] for 1 h at room temperature, and incubated with primary antibodies overnight at 4°C. Secondary antibody incubation was at room temperature for 2 h (Santa Cruz Biotechnology). Chemiluminescence ECL (Amersham, Arlington Heights, IL, USA) was used to detect immunoreactive protein signals. Protein signals were then visualized on films and scanned and quantified using the ImageJ software (National Institutes of Health Image, USA). For re-probing, PVDF membranes were stripped with 0.2 M NaOH for 10 min before blocking with another primary antibody. The expression of interested molecules was determined relative to β-actin.

#### Statistical analysis

Each experiment was repeated at least three times. Quantified results were presented as mean ± S.D. Significant differences were determined using factorial analysis of variance (ANOVA).  $P < 0.05$  was considered significant difference.



**Fig. 1.** Effect of LPS on osteoclast formation. A. TRAP-positive multinucleated cells at 4 d after LPS (0, 10, 50, 100, 200, or 1000 ng/mL) treatment. B. The number of osteoclasts in LPS-treated (0, 10, 50, 100, 200, or 1000 ng/mL) cultures. \*P < 0.05; \*\*P < 0.01. Data represent the mean ± S.D. from three independent experiments.



**Fig. 2.** The number of TRAP-positive multinucleated cells in osteoclasts treated with 5, 10, 15, 25 or 50 μM quercetin in the presence of LPS (100 ng/mL) for A. 24 h and B. 48 h. C. Effect of quercetin treatments (at 15, 25 and 50 μM) on osteoclast survival at 24 and 48 h. \*P < 0.05; \*\*P < 0.01. Data represent the mean ± S.D. from three independent experiments.

## Results

### Quercetin inhibited LPS-induced mature osteoclast survival

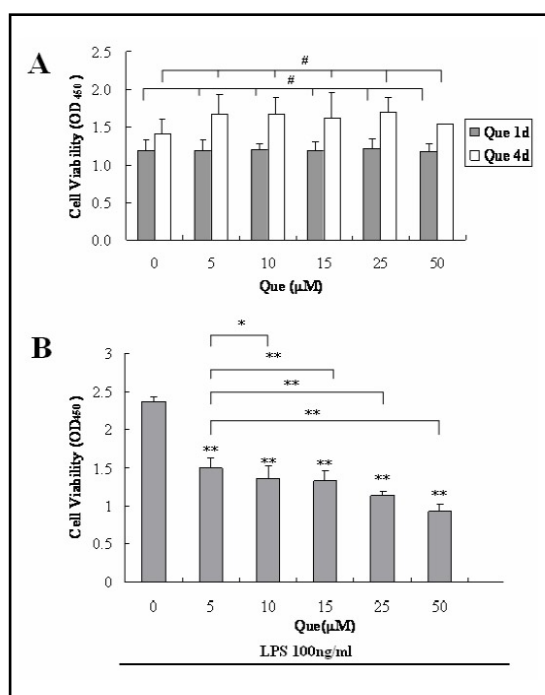
The population of osteoclasts in LPS-treated cultures was significantly higher than that in non-treated cultures. LPS at 100 ng/mL induced the most osteoclast formation among other concentrations of LPS treated cultures (Fig. 1). Further quercetin treatment at 15, 25 and 50 μM significantly decreased the number of osteoclasts induced by LPS (Fig. 2A-C). Quercetin treatment at 50 μM completely suppressed LPS-induced osteoclast formation at 48 h after the addition of quercetin (Fig. 2C).

To ensure the inhibitory effect of quercetin on LPS-induced osteoclast survival, we evaluated cell viability in all treatment groups. Quercetin significantly decreased LPS-induced osteoclast viability in a dose-dependent manner at 24 h after quercetin treatment. Quercetin alone did not induce cytotoxicity in RAW264.7 cells at the concentrations used in the current study (Fig. 3).

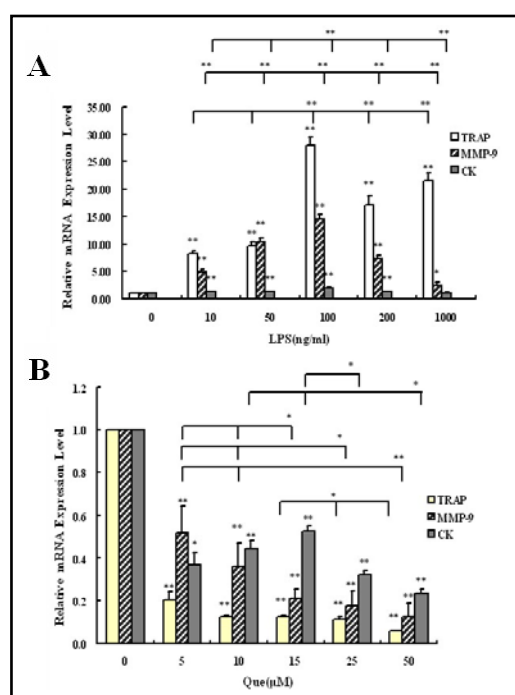
### Quercetin inhibited the mRNA expression of osteoclast-specific genes in mature osteoclasts

On differentiation day 4, LPS at 100 ng/mL significantly increased mRNA expression of *TRAP*, *MMP-9* and *CK* than other concentrations of LPS treated groups as well as non-treated group. Messenger RNA expressions of *TRAP*, *MMP-9* and *CK* in LPS-induced osteoclasts were





**Fig. 3.** Effect of quercetin on cell viability in (A) RAW264.7 cells at 24 h or 4d and (B) LPS-induced mature osteoclasts at 24 h. \* $P < 0.05$ ; \*\* $P < 0.01$ . Data represent the mean  $\pm$  S.D. from three independent experiments.



**Fig. 4.** Messenger RNA expression of *TRAP*, *MMP-9* and *CK* in A. LPS treated (0, 10, 50, 100, 200, or 1000 ng/mL) in RAW264.7 cells at day 4; and B. Quercetin treatment (0, 5, 10, 15, 25, or 50  $\mu$ M) in LPS-induced osteoclasts at 24 h. \* $P < 0.05$ ; \*\* $P < 0.01$ . Data represent the mean  $\pm$  S.D. from three independent experiments.

significantly inhibited in a dose-dependent manner with the treatment of quercetin at 24 h (Fig. 4). Quercetin at 25 and 50  $\mu$ M exhibited complete inhibition on LPS induced mRNA expression of *TRAP* and *MMP-9* in mature osteoclasts.

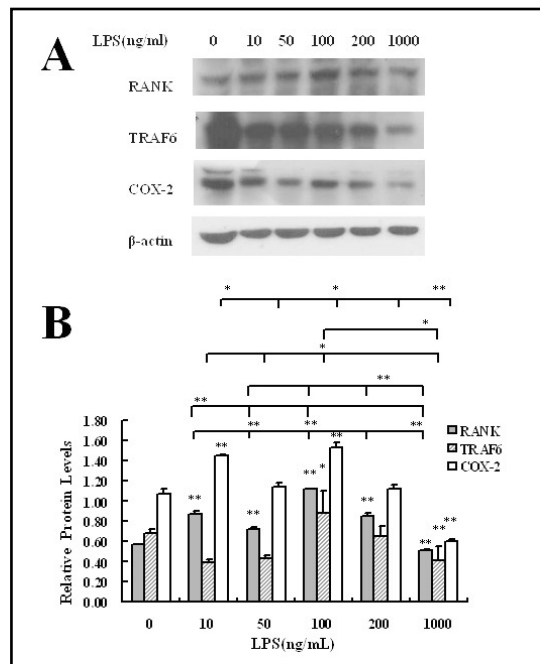
*Quercetin inhibits the expression of RANK, TRAF6 and COX-2 protein in LPS-induced osteoclasts*

LPS at 100ng/mL significantly increased protein expression of RANK, TRAF6 and COX-2; however, higher concentrations of LPS inhibited protein expression of these molecules (Fig. 5). Quercetin significantly inhibited RANK and COX-2 protein expression in a dose-dependent manner when compared to non-quercetin-treated LPS-induced osteoclasts at 24 h ( $P < 0.01$ ) (Fig. 6). Quercetin at 50  $\mu$ M exhibited the highest inhibition on protein expression of TRAF6 when compared to other quercetin concentrations at 24 h (Fig. 6).

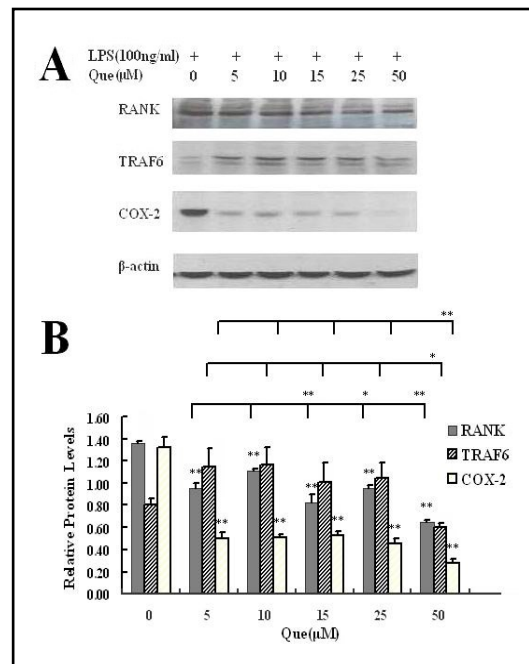
*Quercetin selectively regulates activation of MAPK in LPS-induced osteoclasts*

Since MAP kinases are important regulators of inflammatory mediators and osteoclast differentiation, we used densitometric scanning of Western blots to examine the effect of quercetin on activation of MAPKs in LPS-induced osteoclasts. Quercetin treatment at 50  $\mu$ M enhanced protein levels of phosphorylated forms of p38-MAPK, ERK1/2 and JNK in LPS-induced (100 ng/mL) osteoclasts at 2 h (Fig. 7).

MAPK inhibitors SB203580, PD98059 and SP600125 were added 2 h prior to quercetin treatment and then proteins were prepared 30 min after quercetin treatment in the presence of LPS (100 ng/mL). MAPK inhibitors, SB203580, PD98059 and SP600125, selectively attenuated quercetin-enhanced phosphorylation of p38-MAPK and JNK, but not the phosphorylation of ERK1/2 (Fig. 8). This suggests that quercetin inhibits LPS-induced



**Fig. 5.** A. Protein expression of RANK, TRAF6 and COX-2 after RAW264.7 cells treated with LPS (0, 10, 50, 100, 200, 1000 ng/mL) for 4 d. B. Effect of protein expression of RANK, TRAF6 and COX-2 under LPS at day 4. \*P < 0.05; \*\*P < 0.01. Data represent the mean  $\pm$  S.D. from three independent experiments.



**Fig. 6.** A. Protein expression of RANK, TRAF6 and COX-2 at 24 h after quercetin treatment in LPS-induced osteoclasts. B. Effect of quercetin on protein expression of RANK and COX-2 at 24 h showing in a dose-dependent manner. \*P < 0.05; \*\*P < 0.01. Data represent the mean  $\pm$  S.D. from three independent experiments.

osteoclast formation and bone resorptive activity through selective activations of p38-MAPK, and JNK, both of which are involved in the apoptotic signaling pathway. MAPK inhibitor PD98059 did not attenuate on quercetin-enhanced phosphorylation of ERK1/2. This is possibly because the ERK1/2 signaling pathway inhibits apoptotic processes.

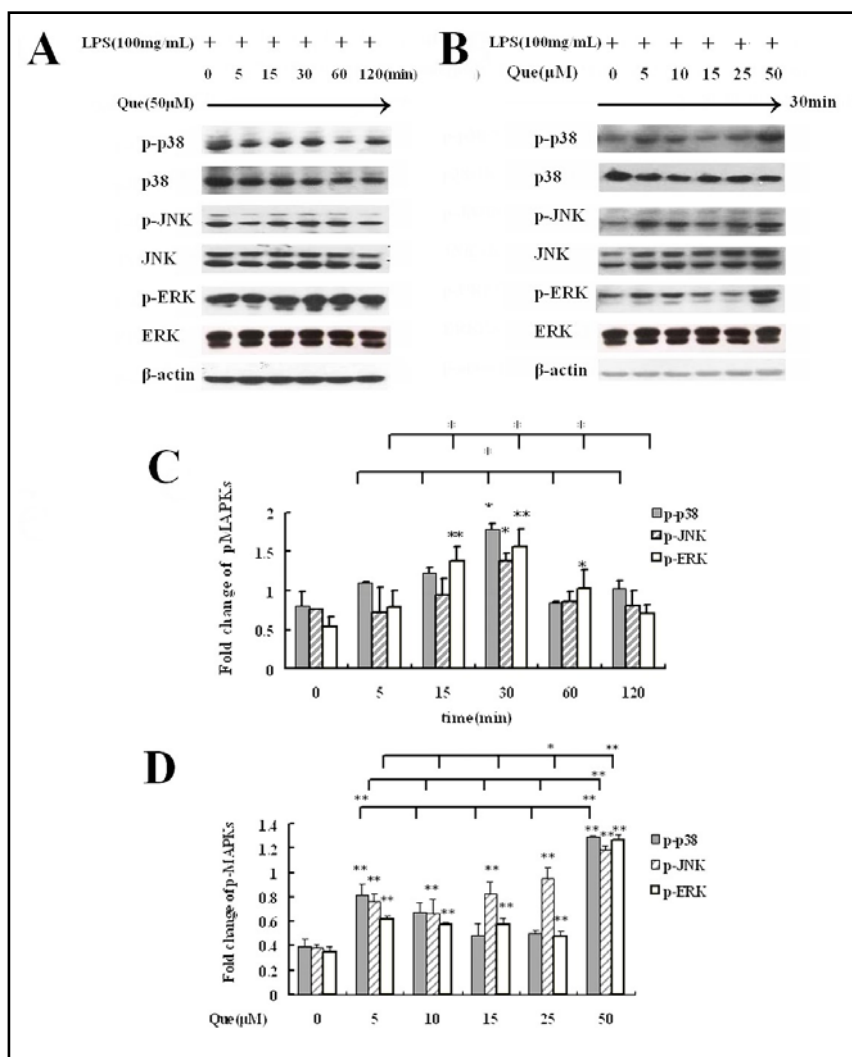
#### Effect of quercetin on cell apoptosis

The effect of exposure to quercetin for 24 h on nuclear condensation was tested using Hoescht fluorescence. After incubation with LPS at 100ng/mL for 4 d, cells were treated with quercetin in the presence of LPS (100ng/mL) for 24 h. Hoechst 33258 staining was analyzed with a fluorescence microscope. Cells with nuclei containing condensed chromatin or cells with fragmented nuclei were defined as apoptotic cells. The number of fluorescence-positive osteoclasts was increased with quercetin treatment in a dose-dependent manner compared to non-quercetin-treated osteoclasts in the presence of LPS at 24 h (Fig. 9). Quercetin at 50  $\mu$ M significantly increased Bax protein expression while inhibiting Bcl-2 protein expression compared to non-quercetin-treated cells in LPS induced osteoclasts (Fig. 10).

#### Quercetin inhibits bone resorptive activity in LPS-induced osteoclasts

RAW264.7 cells were plated on bovine bone slices and incubated with LPS (100 ng/mL) for 6 d. Then administration of quercetin treated into mature osteoclasts in the presence of LPS (100ng/mL) every 3 d. On day 15, bone slices were stained with 1% toluidine blue-1% borate for 5 min. The number of pits in each bone slice was observed and counted using a microscope. The images were also obtained. Quercetin treatments at 15, 25 and 50  $\mu$ M concentrations decreased the number of pits in each bone slice significantly, indicating that osteoclast bone resorption was inhibited as a result of quercetin treatment in the presence of LPS (Fig. 11).

**Fig. 7.** A. and C. Effect of quercetin at 50  $\mu\text{M}$  on the protein expression of MAPKs in LPS-induced osteoclasts at 2 h. Quercetin at 50  $\mu\text{M}$  enhanced activation of MAPKs on 30min ( $P < 0.05$ ). B D Quercetin at 0, 5, 10, 15, 25 or 50  $\mu\text{M}$  was given to LPS-induced osteoclasts for 30 min and the protein expression of MAPKs was examined. Quercetin at 50  $\mu\text{M}$  enhanced the utmost activation of MAPKs ( $P < 0.01$ ). Data represent the mean  $\pm$  S.D. from three independent experiments.

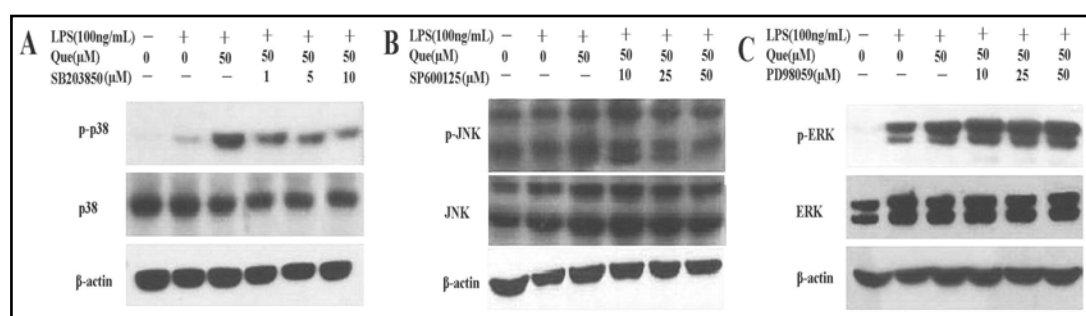


## Discussion

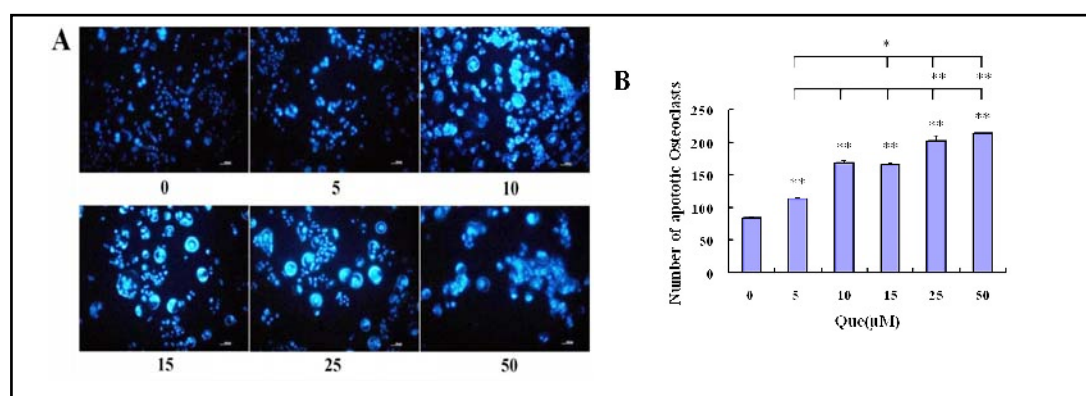
Bacteria-induced inflammatory response is an important contributor to excessive bone resorption in chronic inflammatory diseases such as septic arthritis, osteomyelitis, and infected orthopedic implant failure [16]. LPS, a pro-inflammatory glycolipid and main component of the cell wall of Gram-negative bacteria, is well documented in Gram-negative bacteria-induced bone destruction. LPS stimulates osteoclastic bone resorption *in vivo* [17, 18] and promotes osteoclast differentiation in whole bone marrow cell culture [19] and in preosteoclasts [6, 7]. In the current study, LPS increased the protein expression of RANK, TRAF6 and COX-2 and mRNA expression of *TRAP*, *MMP-9* and *CK* in RAW264.7 cells (100 ng/mL LPS). Compared with LPS-induced osteoclasts, the mRNA expression levels of osteoclast-related genes in RAW264.7 cells were downregulated or unregulated by quercetin (data not shown). These results confirmed that LPS could stimulate osteoclast differentiation directly [6, 7].

Quercetin, as one of the most abundant flavonoids found in onions and other vegetables, has been highlighted as a bioactive substance and could be of potential clinical application in inflammatory diseases [13]. Quercetin has been reported to inhibit osteoclast formation and to prevent bone loss in ovariectomy (OVx) animal models [10-12, 20]. Moreover, *in vivo* evidence show that quercetin has a bone-protecting effect caused by estrogen deficiency, at least in part, by dual regulation of the enhancement of osteoblast function and the induction of osteoclast apoptosis [21]. However, the effect of quercetin on LPS-induced osteoclastogenesis has not yet been reported.





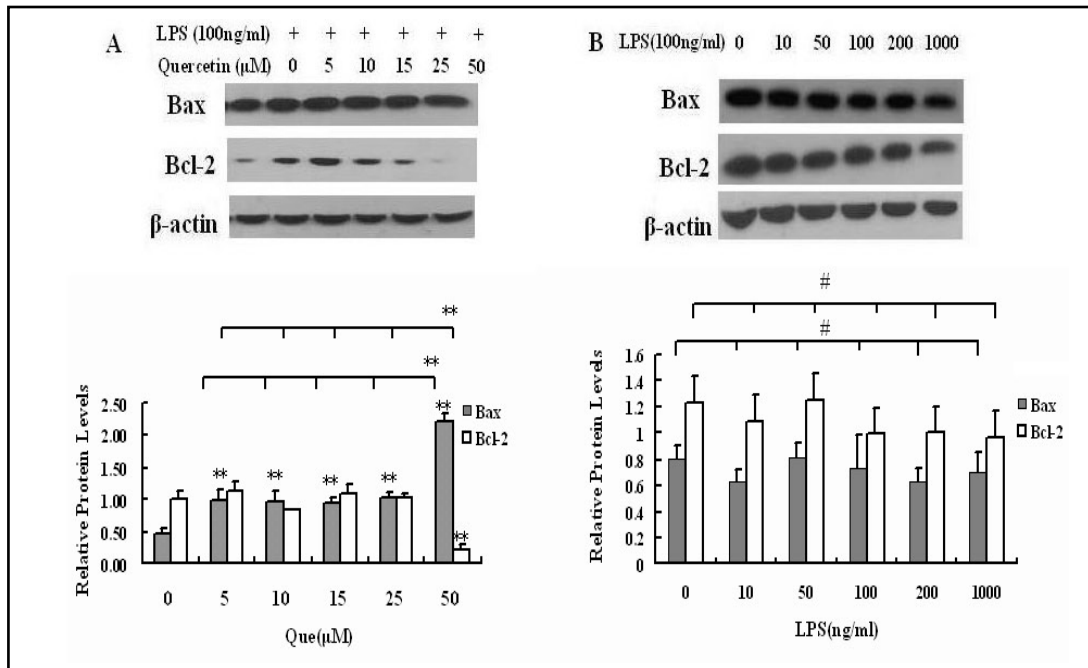
**Fig. 8.** Effect of quercetin on the protein expression and phosphorylation of MAPKs in LPS-induced osteoclasts in the presence of MAPK inhibitors A. SB203580, B. SP600125 and C. PD98059 at 30 min after quercetin treatment. \* $P < 0.05$ ; \*\* $P < 0.01$ . Data represent the mean  $\pm$  S.D. from three independent experiments.



**Fig. 9.** A. Effect of quercetin on nuclei condensation (stained with Hoescht 33258) of mature osteoclasts at 24 h. B. Number of fluorescence-positive osteoclasts induced by quercetin treatment at 24 h (0, 5, 10, 15, 25, or 50  $\mu$ M). \* $P < 0.05$ ; \*\* $P < 0.01$ . Data represent the mean  $\pm$  S.D. from three independent experiments.

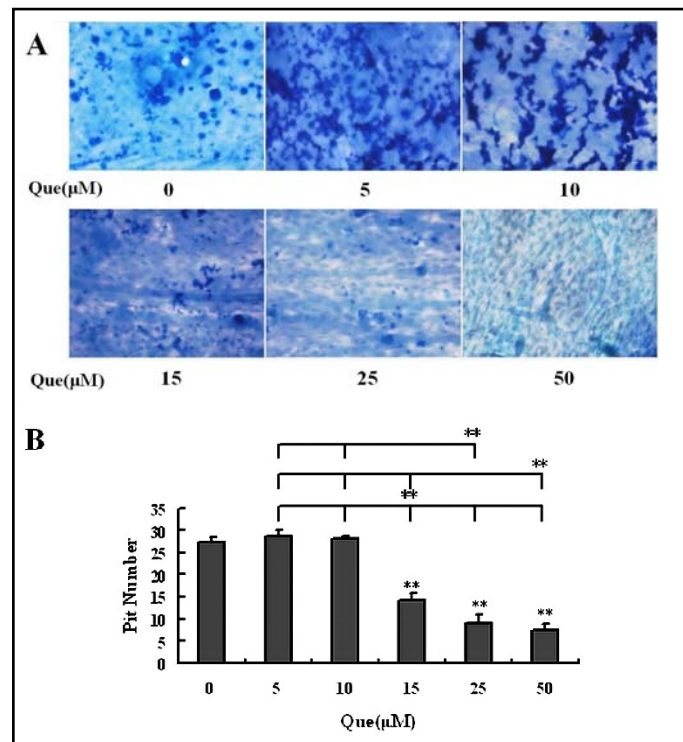
In the current study, we found that quercetin suppressed the expression of osteoclast related molecules, showing decreased mRNA expression of *TRAP*, *MMP-9* and *CK*, as well as decreased protein expression of *RANK*, *TRAF6* and *COX-2* in LPS-induced osteoclasts. On differentiation day 5 and 6, quercetin at 15, 25 and 50  $\mu$ M significantly decreased the number of TRAP-positive multinucleated osteoclasts induced by LPS. Quercetin also significantly decreased LPS-induced osteoclast viability in a dose-dependent manner after 24 h without cytotoxicity in RAW264.7 cells. These data suggest that quercetin suppresses LPS-induced osteoclast survival and bone resorption. Quercetin at 50  $\mu$ M has exerted more potent inhibitory effect on LPS-induced osteoclast survival and bone resorption *in vitro*. The estimated average individual intake of quercetin in the United States is 25 mg per day. In an American diet containing fried onions, quercetin glucosides is about 64 mg, equivalent to 64 mg aglycone, which can reach a maximum serum concentration of 196 ng/mL (equals to 0.6 mM) 2.9 h after ingestion [22]. The same investigators experimented on nine healthy subjects by giving them the amount of quercetin glucosides equivalent to 64 mg aglycone from onions. Plasma levels of quercetin reached the peak of 225 ng/mL (equals to 0.8 mM) after the onion meal [23]. Therefore, in the current report, treatment of quercetin at 50  $\mu$ M (equals to 15.1 ng/mL) is a physiologically relevant dose.

The inhibitory effect of quercetin on LPS-induced osteoclast resorptive activity is confirmed by evaluating mRNA expression levels of osteoclast-related genes. TRAP has been suggested to be involved in bone resorptive activity in osteoclasts [24, 25]. The proteolytic enzymes such as MMP-9 and CK are highly expressed in osteoclasts. They are essential enzymes in degrading the bone matrix during bone resorption. MMP-9 can initiate the bone resorptive process by removing the collagenous layer from the bone surface before



**Fig. 10.** Protein expression of Bax and Bcl-2 in A. LPS-induced mature osteoclasts treated with 0, 5, 10, 15, 25, or 50 μM quercetin at 24h; B. RAW264.7 cells treated with 0, 10, 50, 100, 200 or 1000 ng/mL LPS for 4 d. \*P < 0.05; \*\*P < 0.01. Data represent the mean ± S.D. from three independent experiments.

**Fig. 11.** A. The number of osteoclast bone resorption pits 15 d after quercetin treatments (0, 5, 10, 15, 25, or 50 μM). B. The effect of 15, 25 and 50 μM quercetin on osteoclast bone resorptive activity compared to non-treated culture. \*P < 0.05; \*\*P < 0.01. Data represent the mean ± S.D. from three independent experiments.



demineralization begins [26-28]. CK plays an essential role in osteoclastic bone resorption [29-31]. In the current study, the mRNA expression levels of *TRAP*, *MMP-9* and *CK* as well as bone slices pit in LPS-induced osteoclast were significantly inhibited by quercetin treatment

in a dose-dependent manner. Taken together, our data indicate that the inhibitory effect of quercetin on bone resorptive activity could be due to the inhibition of quercetin on osteoclast-related genes.

Our data show that LPS can induce osteoclast differentiation from RAW264.7 cells on day 4; only from day 6 on the mature osteoclasts were functionally activated. Administration of quercetin to LPS-induced osteoclasts on differentiation day 4 for 24 or 48 h can evaluate the effect of quercetin on osteoclast survival or apoptosis in differentiated osteoclasts. Unreported data from our group also show that quercetin has a potent inhibitory effect on osteoclastogenesis induced by LPS when quercetin is administrated more than 2 h prior to treatment with LPS in RAW264.7 cells.

Induction of apoptosis in mature osteoclasts or suppressing the survival of mature osteoclasts has been suggested to be one of the processes to inhibit osteoclast-mediated bone loss [32]. We further evaluated the effect of quercetin on apoptosis in LPS-induced mature osteoclasts. Our data suggest that quercetin promoted apoptosis in LPS-induced mature osteoclasts in a dose-dependent manner. Moreover, quercetin promoted protein expression of Bax, while inhibiting Bcl-2. Based on these results, we conclude that the inhibitory effect of quercetin on bone resorptive activity also involves the induction of apoptosis in LPS-induced osteoclasts.

MAPKs are activated by a variety of stresses including LPS and then affect apoptosis either in positive or negative manners [33]. In many cell types, JNK and p38-MAPK contribute to the induction of apoptosis, whereas ERK1/2 inhibits apoptotic processes [34-36]. In the current study, treatment with quercetin enhanced phosphorylation of ERK1/2, p38-MAPK and JNK. However, re-treatment with MAPK inhibitors SB203580, PD98059 and SP600125 alone attenuated quercetin-enhanced phosphorylation of p38-MAPK and JNK, but not ERK1/2. These data suggests that quercetin can promote apoptosis in mature osteoclast through the p38-MAPK and JNK signaling pathways, which further inhibit LPS-induced osteoclast resorptive activity.

COX-2 plays a critical role in a number of osteoclastogenic signaling pathways [37, 38]. Inhibition of COX-2 blocks osteoclast formation *in vitro* [39, 40]. In the current study, LPS upregulated the protein expression of COX-2 in RAW264.7 cells, whereas quercetin inhibited COX-2 expression in LPS-induced osteoclasts, indicating that the inhibitory effect of quercetin on COX-2 expression in LPS-induced osteoclasts also results in blocking LPS-induced osteoclastogenesis. Our results agreed with previous publications [39, 40].

RANK signal transduction has been reported to be essential for osteoclast differentiation, bone resorptive function, and survival [41, 42]. Mice deficient in *RANKL* or *RANK* genes exhibit severe osteopetrosis because they do not form osteoclasts [43-46]. Selective inhibition of RANK with RANK:Fc or RANK receptor inhibitor blocks osteoclast maturation and function *in vivo* or *in vitro* [47-50]. Selective inhibition of TRAF6 also blocks osteoclast maturation and function [41, 51]. Our results show that LPS enhanced protein expression of RANK and TRAF6 in RAW264.7 cells. However, quercetin can inhibit RANK protein expression in a dose-dependent manner, while quercetin at 50  $\mu$ M decreases the protein expression of TRAF6 at 24 h. This suggests that RANK/TRAF6 signaling plays a prominent role in osteoclastogenesis downstream of LPS, and quercetin inhibits mature osteoclast activities via RANK signal transduction.

In conclusion, our data suggest that quercetin can inhibit LPS-induced osteoclastogenesis and bone resorption. Our findings indicate that quercetin could be an effective drug to treat human abnormal bone loss induced by LPS in chronic inflammatory diseases.

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