Puerarin Prevents LPS-Induced Osteoclast Formation and Bone Loss via Inhibition of Akt Activation

Yun Zhang,*a,† Ming Yan,*b,† Qing-feng Yu,a Pei-fan Yang,a Hai-dong Zhang,a Yong-hong Sun,c Zhi-fen Zhang,a and Yun-feng Gaoa

*a College of Medicine, Shaoxing University; Huancheng West Road 508, Shaoxing 312000, China; b Department of Biomedical Engineering, College of Life Information Science and Instrument Engineering, Hangzhou Dianzi University; 2nd Avenue 1158, Xiasha Higher Education Zone, Hangzhou 310018, China; and c Zhejiang Provincial Key Laboratory of Cardio-Cerebral Vascular Detection Technology and Medicinal Effectiveness Appraisal, Department of Biomedical Engineering, Zhejiang University; Hangzhou 310027, China.

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Osteolysis induced by chronic Gram-negative bacterial infection underlies many bone diseases such as osteomyelitis, septic arthritis, and periodontitis. Drugs that inhibit lipopolysaccharide (LPS)-induced osteolysis are critically needed for the prevention of bone destruction in infective bone diseases. In this study, we assessed the effect of puerarin, a natural isoflavone isolated from Pueraria lobata Ohwi root, on LPS-induced osteoclastogenesis and bone loss. Our in vitro study showed that puerarin significantly inhibited LPS-induced osteoclast differentiation from osteoclast precursor RAW264.7 cells. The inhibition occurred through suppressing the production of osteoclast activating factor tumor necrosis factor (TNF)-α, interleukin (IL)-1β and prostaglandin E2 (PGE2), which led to down-regulating mRNA expression of osteoclastogenic genes including tartrate-resistant acid phosphatase (TRAP), cathepsin K and matrix metalloprotein 9 (MMP-9). Furthermore, LPS triggered activation of Akt in osteoclast precursor RAW264.7 cells, which was inhibited by puerarin treatment. In vivo, puerarin attenuated LPS-induced bone loss in a murine calvarial osteolysis model. Collectively, puerarin prevents LPS-induced osteoclast formation, function and bone loss, where the inhibition of Akt activation plays an important role. These findings provide evidences that puerarin might be beneficial as a promising candidate drug for the prevention and treatment of bacteria-induced bone destruction disease, and give new insights for understanding its possible mechanism.

Key words puerarin; lipopolysaccharide (LPS); osteoclastogenesis; bone loss; Akt

Osteoclast is formed by differentiation and fusion of hematopoietic stem cells, with the unique capacity to resorb bone by either enhancing the differentiation of osteoclast precursor or maturation of multinucleated cell. It plays a key physiological role in bone remodeling and a pathological role in bone destruction diseases including osteopetrosis, osteoporosis, rheumatoid arthritis and inflammatory osteosclerosis. Bacteria and their products (such as lipopolysaccharide (LPS)) can induce inflammatory bone loss, leading to significant morbidity and substantial health care expenditure. LPS, a bacteria-derived cell wall product, is recognized as a key pathogen of inflammatory osteolytic diseases such as osteomyelitis, septic arthritis, periodontitis, and infection of orthopedic implants. LPS induces the production of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, prostaglandin E2 (PGE2) and IL-6, that are able to directly stimulate osteoclast differentiation and ultimately lead to the destructive bone loss via increasing the expression of receptor activator for nuclear factor-κB ligand (RANKL). RANKL is the key cytokine that stimulates entire processes for the development of bone-resorbing osteoclasts. The binding of RANKL to its receptor RANK triggers the activation of signaling molecules such as Akt that subsequently induce the activation of transcription factors to regulate the expression of genes required for osteoclast differentiation and survival. To date, effective therapy against LPS-induced bone destruction is limited to antibiotics and surgery, which may cause undesirable complications and serious surgical trauma. Therefore, to identify drugs that inhibit LPS-induced osteolysis is a major goal in the treatment of local and systemic bone loss in chronic inflammatory diseases.

Pueraria lobata Ohwi (Wild) root is widely used in China, Japan and Korea for the treatment of osteoporosis and myocardial infarction with rare side effects. Puerarin (daidzein 8-C-glucoside, Fig. 1), the main isoflavone glycoside and the...
major active ingredient extracted from *Pueraria lobata* (Wild) root, has beneficial effects in possessing anti-oxidative stress, anti-cerebral ischemia, anti-diabetes, anti-atherosclerosis, anti-myocardial ischemia and anti-apoptosis properties in the clinical practice. Several pre-clinical studies showed that puerarin inhibits inflammation and regulates the release of inflammatory mediators like TNF-α, IL-6 and IL-1β, and it also ameliorates LPS or tetrachloride-induced kidney injury in mice by inhibiting inflammatory response. These results suggest that puerarin exhibiting anti-inflammatory activity, may affect LPS-induced the production of pro-inflammatory cytokines, which enhance osteoclast differentiation and bone loss. In addition, puerarin has been reported to effectively inhibit bone loss in rats with estrogen deficiency or with ligature-induced periodontitis. However, the effect of puerarin on LPS-induced inflammatory bone loss remains unknown.

In the present study, we observed the *in vitro* effect of puerarin on LPS-induced osteoclast differentiation and function, as well as the production of pro-inflammatory cytokines like TNF-α, IL-6 and IL-1β. To illustrate its molecular mechanism, we further explored Akt signaling after puerarin treatment in the presence or absence of LPS. *In vivo*, we investigated the potential efficacy of puerarin to alleviate LPS-induced inflammatory calvarial osteolysis in a mouse model.

**MATERIALS AND METHODS**

**Chemical and Reagents** *Escherichia coli* LPS (serotype 055:B5), leukocyte Acid Phosphatase Kit 387-A, 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and enzyme-linked immunosorbent assay (ELISA) kits of TNF-α, IL-1β and PGE2 were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), SuperScript® First Strand Synthesis System for real time (RT)-PCR were supplied by Invitrogen (Carlsbad, CA, U.S.A.). TRIzol® and Chemiluminescent Substrate (ATCC). Cells were maintained in DMEM medium supplemented with 10% FBS and 1% (v/v) penicillin-streptomycin solution and incubated at 37°C in 5% CO2 humidified air. RAW264.7 cells were cultured in the presence of LPS (0.1 µg/mL) for 72 h.

**Osteoclast Differentiation and Tartrate-Resistant Acid Phosphatase (TRAP) Staining** For osteoclast differentiation, RAW264.7 cells were treated with puerarin (10–50 µmol/L) for 72 h in the presence or absence of LPS (0.1 µg/mL). Cells were washed twice with phosphate buffered saline (PBS), fixed in 4% paraformaldehyde (pH 7.4) at room temperature (r.t.) for 15 min and stained for TRAP using leukocyte Acid Phosphatase Kit 387-A according to the manufacturer's protocol. TRAP-positive multinucleated cells (MNCs) containing three or more nuclei were counted as osteoclast under a IX70 microscope (Olympus, Japan).

**Cell Viability Assay** The cell viability was observed by the fluorescence of Calcein-AM staining in viable cell. In brief, RAW264.7 cells cultured on the coverslip were treated puerarin (10–100 µmol/L) for 48 h. The cells were washed twice in PBS, and then incubated with Calcein-AM (5 µmol/L) for 15 min at 37°C. The grafts were washed and visualized with a LSM 510 confocal laser microscope (Carl Zeiss, Germany) at excitation/emission wavelength of 488/517 nm.

In addition, MTT was used to quantitatively measure the effect of puerarin on the viability of RAW264.7 cells. The assay is a colorimetric method for measuring the number of viable cells in proliferation or cytotoxicity. RAW264.7 cells were cultured in 96-well treated with or without puerarin (10–100 µmol/L) for 48 h. Ten microliters of MTT (5 mg/mL) in PBS was added per well and cells were further incubated for 4 h at 37°C. Then MTT medium was removed and 200 µL dimethyl sulfoxide (DMSO) was added. The absorbance was measured at 490/570 nm under Multiskan Spectrum Microplate Reader (Thermo Scientific, Waltham, U.S.A.).

**Analyses of Pro-inflammatory Cytokines** RAW264.7 cells were culture for 24 h, and treated with puerarin (10–50 µmol/L) plus LPS (0.1 µg/mL) for 24 h. Culture supernatants were collected and frozen at −80°C. The levels of TNF-α, IL-1β and PGE2 in culture supernatants were determined using commercial ELISA detection kits according to the manufacturer's instructions.

**Table 1. Sequence of PCR Primers Used for Real-Time Quantitative PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Sequences (5′→3′)</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRAP</td>
<td>NM_007388</td>
<td>CACTCCACCATGTAGATTTTGT (F)</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CATCGTCTGACGGTTTCTG (R)</td>
<td></td>
</tr>
<tr>
<td>Cathepsin K</td>
<td>NM_007802</td>
<td>CTTAAGATGTCTTCCCCATATGTGGG (F)</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCAAGCGTGTCTCTATCCGAGC (R)</td>
<td></td>
</tr>
<tr>
<td>MMP-9</td>
<td>NM_013599</td>
<td>CTGGAACGCGACAACTAAAAG (F)</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTCGGGCGAAGTCTTCCAGAG (R)</td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>NM_007393</td>
<td>GGCTGTATCTCCCTCATCCG (F)</td>
<td>154</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCAGTTGGTAAACATGGCCATGT (R)</td>
<td></td>
</tr>
</tbody>
</table>
RNA Extraction and Quantitative RT-PCR Assay

After RAW264.7 cells were cultured for 24 h, cells were treated with puerarin (10–50 \( \mu \text{mol/L} \)) plus LPS (0.1 \( \mu \text{g/mL} \)) for 72 h, and total RNA was extracted by a standard protocol using TRIzol® reagent. Reverse-transcription was performed to generate first-strand DNA and used for the PCR template. Quantitative gene expression analyses were carried out using real-time PCR by means of the SYBR® Green stain and the Smart Cycler® system as described previously. The primers using TRIzol® reagent.

Reverse-transcription was performed for 72 h, and total RNA was extracted by a standard protocol. After RAW264.7 cells were cultured for 24 h, cells were treated with LPS (0.1 \( \mu \text{g/mL} \)) for 72 h. The cellular lysates were prepared as described previously. The proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically onto polyvinylidifluoride (PVDF) membranes. The blots were blocked with 4% bovine serum albumin (BSA) for 1 h at r.t. and then probed with rabbit anti-mouse antibodies against Total Akt, p-AktSer473, p-AktThr308 and \( \beta \)-actin at 4°C overnight. After three washes, the blots were subsequently incubated with a rabbit anti-mouse horseradish peroxidase (HRP) conjugated secondary antibody for 2 h at r.t. After four times washing for 10 min in Tris-buffrer saline with Tween 20 (TBST), the membranes were detected using an chemiluminescence detection system. The intensity of the bands was quantified by scanning densitometry using the National Institutes of Health Image 1.61 software.

Western Blot Analysis

RAW264.7 cells were cultured for 24 h, and treated with LPS (0.1 \( \mu \text{g/mL} \)) for 12, 24 and 48 h, or with puerarin (10–50 \( \mu \text{mol/L} \)) plus LPS (0.1 \( \mu \text{g/mL} \)) for 48 h. The cellular lysates had been utilized in previous reports. 17–19) PBS, with or without puerarin (1 mg/kg/d) was then locally injected into the calvaria for two weeks. All mice survived until the experiment ended, and were then sacrificed in a CO\(_2\) chamber. The calvaria were collected and fixed with 4% paraformaldehyde for 10 min, and then were stained with TRAP, and osteolysis area was analyzed by NIH Image J.

Statistical Analysis

Results are expressed as the mean±standard deviation (S.D.). Statistical comparisons between groups were carried out by one-way ANOVA to compare differences between experimental and control group. The least significance difference (LSD) test was used for multiple comparisons. \( p \) values <0.05 were considered significant.

RESULTS

Puerarin Inhibited LPS-Induced Osteoclast Formation

To observe the effect of puerarin on LPS-induced osteoclastogenesis, RAW264.7 cells were treated with puerarin (10–50 \( \mu \text{mol/L} \)) for 72 h in the presence or absence of LPS, and stained with TRAP, a osteoclast-specific gene. Data showed that LPS can directly stimulate osteoclast precursor RAW264.7 cells differentiation into osteoclast, and the maximal number and size of TRAP(+)MNCs was observed at 0.1 \( \mu \text{g/mL} \) of LPS incubation for 72 h (data not shown). Puerarin treatment caused osteoclast less rounded and few nuclei than that of LPS-treated group (Fig. 2A), and dose-dependently inhibited LPS-induced osteoclast formation (Fig. 2B). Notably, 50 \( \mu \text{mol/L} \) of puerarin completely inhibited LPS-induced osteoclast formation, and the number of TRAP(+)MNCs/well was reduced to 38.6% of osteoclast formed in LPS-treated group. Whereas, puerarin at 10 \( \mu \text{mol/L} \) inhibited osteoclastogenesis less efficiently than the other puerarin-treated groups (Fig. 2A). As TRAP(+) multinucleated osteoclasts (MNCs) were stained with TRAP, a osteoclast-specific gene. Data showed that LPS can directly stimulate osteoclast precursor RAW264.7 cells differentiation into osteoclast, and the maximal number and size of TRAP(+)MNCs was observed at 0.1 \( \mu \text{g/mL} \) of LPS incubation for 72 h (data not shown). Puerarin treatment caused osteoclast less rounded and few nuclei than that of LPS-treated group (Fig. 2A), and dose-dependently inhibited LPS-induced osteoclast formation (Fig. 2B). Notably, 50 \( \mu \text{mol/L} \) of puerarin completely inhibited LPS-induced osteoclast formation, and the number of TRAP(+)MNCs/well was reduced to 38.6% of osteoclast formed in LPS-treated group. Whereas, puerarin at 10 \( \mu \text{mol/L} \) inhibited osteoclastogenesis less efficiently than the other puerarin-treated groups (Fig. 2A).

(A) After RAW264.7 cells were cultured for 24 h, cells were treated with puerarin (10–50 \( \mu \text{mol/L} \)) plus LPS (0.1 \( \mu \text{g/mL} \)) for 72 h. Cells were stained for TRAP(+) multinucleated cells (MNCs) containing more than three nuclei were counted as osteoclast under IX70 microscope. The maximal number and size of TRAP(+)MNCs containing more than three nuclei were counted as osteoclast under IX70 microscope. (B) Data were expressed as TRAP(+)MNCs/well (the mean±S.D.). *\( p < 0.05 \) compared with control group; **\( p < 0.05 \) compared with LPS-treated group. Bar=50 \( \mu \text{m} \).
groups. To exclude the inhibitory effects of puerarin on osteoclastogenesis could have been due to its cytotoxicity on osteoclast precursor RAW264.7 cells, calcein-AM staining and MTT assay were performed. Data indicated that puerarin at 10–50 μmol/L caused neither cytotoxicity nor reduced growth of osteoclast precursors in this study (Fig. 3).

Puerarin Suppressed the Release of Pro-inflammatory Cytokines Exposure of macrophages to LPS induces their activation and the secretion of pro-inflammatory cytokines via a TLR4 dependent pathway. These cytokines enhance osteoclast formation and migration in bone destructive diseases such as rheumatoid arthritis and periodontitis. Consistent with this, RAW264.7 cells incubated with LPS for 24 h resulted in obvious increases in TNF-α, IL-1β and PGE₂ (Table 2), and their levels were increased to 29.17-, 16.76- and 7.60-fold of untreated control group, respectively. Puerarin treatment for 24 h suppressed LPS-induced the production of these cytokines (Table 2) with a dose-dependent manner. Importantly, puerarin at 50μmol/L caused a significant reduction of TNF-α, IL-β and PGE₂, with 2875.49±36.40, 89.56±10.12 and 186.85±20.34 pg/mL, which were much lower than that of LPS-treated group.

Puerarin Down-Regulated mRNA Expression of Osteoclastogenic Genes during Osteoclast Differentiation To further analyze the effect of puerarin on osteoclast differentiation, RAW264.7 cells were treated with puerarin in the presence or absence of LPS, and mRNA expression of osteoclast differentiation-related genes that encode TRAP, cathepsin K and MMP-9 were analyzed. Quantitative RT-PCR data indicated that LPS at 0.1 μg/mL significantly upregulated mRNA expression of TRAP, cathepsin K and MMP-9 during osteoclast formation (Table 3). Puerarin treatment for 72 h obviously down-regulated mRNA expression of these osteoclastogenic genes, and the inhibitory effects were in dose-dependent manner (Table 3). Particularly, 50 μmol/L of puerarin exhibited the inhibition on these genes more stronger than those of the other two concentrations, with their levels were reduced to 22.6% (for TRAP expression), to 31.1% (for cathepsin K expression) and to 29.4% (for MMP-9 expression), respectively (Table 3). However, the effect of lower concentration of puerarin (at 10μmol/L) on mRNA expression of these genes was not different from the untreated control group.

Puerarin Inhibited LPS-Triggered Activation of Akt To elucidate the molecular mechanisms underlying the in-

### Table 2. Puerarin Suppressed LPS-Induced the Production of TNF-α, IL-1β and PGE₂ (n=4)

<table>
<thead>
<tr>
<th>Groups</th>
<th>TNF-α (pg/mL)</th>
<th>IL-1β (pg/mL)</th>
<th>PGE₂ (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>213.61±23.64</td>
<td>23.43±11.29</td>
<td>156.27±4.63</td>
</tr>
<tr>
<td>LPS</td>
<td>623.42±55.12*</td>
<td>392.76±27.16*</td>
<td>1187.56±16.30*</td>
</tr>
<tr>
<td>LPS+Puerarin 10 μmol/L</td>
<td>5102.36±51.45*</td>
<td>336.40±23.62*</td>
<td>924.63±74.16*</td>
</tr>
<tr>
<td>LPS+Puerarin 25 μmol/L</td>
<td>3496.02±46.72*</td>
<td>294.17±19.44*</td>
<td>453.71±34.53*</td>
</tr>
<tr>
<td>LPS+Puerarin 50 μmol/L</td>
<td>2875.49±36.40*</td>
<td>89.56±10.12*</td>
<td>186.85±20.34*</td>
</tr>
</tbody>
</table>

Data were expressed as the mean±S.D. *p<0.05 compared with control group; †p<0.05 compared with LPS-treated group.

### Table 3. Puerarin Decreased mRNA Expression of Osteoclastogenic Genes during Osteoclast Differentiation (n=3)

<table>
<thead>
<tr>
<th>Groups</th>
<th>TRAP (mRNA expression)</th>
<th>Cathepsin K (mRNA expression)</th>
<th>MMP-9 (mRNA expression)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.232±0.05</td>
<td>0.361±0.06</td>
<td>0.164±0.09</td>
</tr>
<tr>
<td>LPS</td>
<td>1.000±0.07*</td>
<td>1.000±0.09*</td>
<td>1.000±0.11*</td>
</tr>
<tr>
<td>LPS+Puerarin 10 μmol/L</td>
<td>0.561±0.05*</td>
<td>0.692±0.04*</td>
<td>0.773±0.02*</td>
</tr>
<tr>
<td>LPS+Puerarin 25 μmol/L</td>
<td>0.373±0.03*</td>
<td>0.435±0.05*</td>
<td>0.392±0.03*</td>
</tr>
<tr>
<td>LPS+Puerarin 50 μmol/L</td>
<td>0.226±0.02*</td>
<td>0.311±0.01*</td>
<td>0.294±0.02*</td>
</tr>
</tbody>
</table>

Data were expressed as the mean±S.D. *p<0.05 compared with control group; †p<0.05 compared with LPS-treated group.
hibitory effect of puerarin on LPS-induced osteoclast differentiation, we explored whether puerarin affects Akt activation in response to LPS. As shown in Fig. 4, stimulation of RAW264.7 cells with LPS significantly triggered activation of Akt at the phosphorylation site Ser473/Thr308, and the increased levels of p-AktSer473 and p-AktThr308 were observed at 12, 24 and 48h. Furthermore, LPS triggered time-course of Akt activation at the phosphorylation site Thr308, and the level of p-AktThr308 was increased about 10-fold of the untreated control group.

Inhibition of Akt activation can inhibit osteoclast differentiation, which prevents osteoclastogenesis and bone loss in infection or in LPS environment. As expected, we found that LPS-triggered Akt activation was significantly inhibited by puerarin treatment, and the levels of p-AktThr308 and p-AktSer473 were obviously decreased (Fig. 5A). Notably, treatment with puerarin for 48h dose-dependently inhibited Akt activation at the phosphorylation site Thr308 more significantly, and densitometric analysis revealed that the level of p-AktThr308 was decreased from 33.5 to 21.05 (10\(\mu\)mol/L), to 20.9 (25\(\mu\)mol/L), and to 7.71 (50\(\mu\)mol/L), respectively (Fig. 5B).

**Puerarin Prevented LPS-Induced Bone Loss in Vivo**

To address the effect of puerarin in vivo, we established osteolytic mouse model by LPS injection as previously described. Mice were intraperitoneally injected with LPS with or without puerarin. No fatalities were recorded after LPS and puerarin administration, and the animals retained normal activity throughout the duration of the experiment. Histological examination confirmed the protective effects of puerarin on LPS-induced bone loss (Table 4, Fig. 6). Injection of LPS locally into the subcutaneous tissue over the calvaria of mice resulted in a large number of osteoclast formed and severe osteolysis, as confirmed by the distinct increase in osteoclast number and the extensive eroded area on the mouse calvaria (Fig. 6). Compared to the untreated control mice, LPS injection significantly increased the ratio of osteoclast number and bone resorption by 5.78- and 5.82-fold (Fig. 6B), suggesting that calvarial osteolysis had occurred in the LPS-injected group. Puerarin (1 mg/kg) treatment reduced the extent of LPS-induced osteoclastogenesis and bone loss (Fig. 6A), and osteoclast number and area of calvarial osteolysis were decreased.

![Fig. 4. LPS Triggered Activation of Akt in Osteoclast Precursor RAW264.7 Cells (n=3)](A) RAW264.7 cells were incubated with LPS (0.1\(\mu\)g/mL) for the indicated times, and cell lysates were prepared and analyzed by Western blot with specific antibodies to detect the levels of p-Akt and Total Akt. (B) Densitometer was analyzed by image analyzing software, and data were expressed as the relative level of p-Akt. *p<0.05 compared with control group (p-AktSer473); #p<0.05 compared with control group (p-AktThr308).

![Fig. 5. Puerarin Inhibited LPS-Induced Activation of Akt in Osteoclast Precursor RAW264.7 Cells (n=4)](A) RAW264.7 cells were cultured for 24h, and treated with puerarin (10–50\(\mu\)mol/L) plus LPS (0.1\(\mu\)g/mL) for 48h. Cell lysates were prepared and analyzed by Western blot with specific antibodies to detect the levels of p-Akt and Total Akt. (B) Densitometer was analyzed by image analyzing software, and data were expressed as the relative levels of p-Akt. *p<0.05 compared with control group; #p<0.05 compared with LPS-treated group.

<table>
<thead>
<tr>
<th>Table 4. Clinical Characteristics</th>
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<tbody>
<tr>
<td>Groups</td>
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</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>LPS</td>
</tr>
<tr>
<td>Puerarin (1 mg/kg/d)</td>
</tr>
</tbody>
</table>

Data are expressed as the mean±S.D. *p<0.05 compared with control group; #p<0.05 compared with LPS-treated group.
creased to 9.43±2.06 (per 0.25 mm interval calvaria sections),
and to 0.117±0.091 mm², respectively (Figs. 6B, C). These
results further identified puerarin protective effect on inflam-
matory bone loss.

DISCUSSION

LPS, the principal component of Gram-negative bacte-
rial cell walls, is a stimulator of osteoclastogenesis and bone
resorption in inflammatory bone destruction. Our data and
previous study showed that LPS could trigger osteoclast pre-
cursor RAW264.7 cells to release pro-inflammatory cytokines
TNF-α, IL-1β and PGE₂, that directly stimulate osteoclast
differentiation, and increase excessive bone resorption. 4,5) Fur-
thermore, we demonstrated that LPS dramatically increased
mRNA expression of osteoclast-specific genes such as TRAP,
cathepsin K and MMP-9 (Table 3), involved in degrading the
bone matrix during bone resorption by initiating the bone re-
sorptive process, or by removing the collageous layer from the
bone surface before demineralization begins. Combining these
previous reports and our results, we conclude that LPS can
directly induce osteoclastogenesis independent of RANKL,
and afterwards cause bone resorption in inflammatory bone
destruction diseases like osteomyelitis, septic arthritis, peri-
odontitis.

Currently, researchers began to focus on identification of
osteoclast targeted-agents due to its potential inhibition of
LPS-induced osteoclastogenesis and subsequent pathological
bone loss. Puerarin, a natural isoflavones from Pueraria lo-
obata Ouwi (Wild) root, has been widely used in the prevention
and treatment of hypertension, coronary artery heart disease,
cerebral infarction and diabetes. Some preclinical in vivo and
in vitro studies reported that puerarin was able to enhance
new bone formation, 22–24) and prevent bone loss through an es-
tragen-like effect. 10,25,26) Consistent with their results, puerarin
inhibited LPS-induced TRAP(+)MNCs formation in a dose-
dependent manner (Fig. 2), and the maximal concentration for
inhibiting osteoclast formation at 50 µmol/L was higher than
that of genistein (0.01–1 µmol/L) or daidzein (0.1–10 µmol/L)
on osteoclastogenesis induced by TNF-α or RANKL in the
same cells, respectively. 27,28) These observations indicate that
although the inhibition of puerarin is a little weaker than that
of structurally similar isoflavones such as genistein and daid-
zein at the same concentrations, it does inhibit LPS-induced
osteoclast formation as well as the resorption activity of ma-
ture osteoclast.

 Besides promotion of osteoclast formation, LPS can also
increase the production of pro-inflammatory cytokines, which
are responsible for osteoclast differentiation, and eventually
inflammatory bone destruction. For example, some previous
reports suggest that TNF-α, IL-1β and PGE₂ are the major
pro-inflammatory mediators provoking osteolysis directly or
indirectly; and these inflammatory factors can be produced
from the destruction of gingival tissue during bacterial infec-
tion. Therefore, it is worthy to study whether the osteoclast
targeted-agents such as puerarin could inhibit inflammatory

![Fig. 6. Puerarin Prevented LPS-Induced Osteoclastogenesis and Osteolysis in Vivo)](image-url)
bone destruction by reduction of pro-inflammatory cytokines. Several reports noted that isoflavones (coumestrol, daidzein and genistein) showed a direct suppressive effect on cytokine-induced osteoclast differentiation.\textsuperscript{27,28} To agree with their results, treatment with puerarin (10–50 µmol/L) effectively inhibited the production of the inflammatory mediators (TNF-α, IL-1β, PGE\textsubscript{2}) induced by LPS (Table 2). Interestingly, puerarin (at 10 µmol/L) had only an obvious inhibitory effect on TNF-α production induced by LPS (Table 2), which is not enough to prevent LPS-induced osteoclastogenesis. The results were consistent with the inhibitory effect of puerarin on TRAP(+)MNCs formation (Fig. 2). Taken together, puerarin can suppress LPS-induced the production of these pro-inflammatory cytokines, which prevents later stages of osteoclast differentiation in the infective bone destruction.

Our results together with previous studies indicated that Akt was activated by LPS (Fig. 4) or adenoviruses infection\textsuperscript{7,8}; and it serves as a central role in the regulation of osteoclast differentiation and survival. Blockade of Akt activation by LY294002 or Akt silencing resulted in the inhibition of osteoclast differentiation via regulation of RANKL-induced activation of nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1).\textsuperscript{29,30} Our Western blot analysis showed that puerarin significantly inhibited LPS-induced activation of Akt (Fig. 5), which are critical for osteoclast differentiation (Fig. 3). Interestingly, puerarin at 50 µmol/L inhibited Akt activation at the phosphorylation site of Ser473, similar to its effect on Thr308 site, but puerarin below this concentration had no obvious inhibition on the Ser473 site. These differences may be due to Akt activation by LPS at the Thr308 site before the site of Ser473. These data indicate that Akt signaling is involved in the puerarin' inhibition on LPS-induced osteoclast formation.

Consistent with the in vitro study, administration of puerarin inhibited bone loss induced by LPS, through the suppression of osteoclastogenesis and function (Table 4, Fig. 6). However, we cannot exclude the possibility that puerarin might affect osteoblastic bone formation. Further studies on the influence of puerarin on osteoblasts might provide more insight into its inhibitory effect on osteoclastogenesis. In summary, puerarin can inhibit LPS-induced osteoclast formation and bone resorption via inhibition of Akt activation. Our findings suggest a novel therapeutic approach to control bacteria-induced bone destruction disease, and demonstrate Akt signaling may be another pathway for puerarin’s blockade of osteoclast differentiation in the infective bone destruction.

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**Conflict of Interest** The authors declare no conflict of interest.

**REFERENCES**


