



ORIGINAL ARTICLE

# Effects of *Salvia miltiorrhiza* ethanolic extract on lipopolysaccharide-induced dental alveolar bone resorption in rats



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## KEYWORDS

inflammation;  
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**Abstract** *Background/purpose:* *Salvia miltiorrhiza* (SM) Bunge (Labiatae/Lamiaceae; common name danshen) is a Chinese medicine that improves blood circulation and inhibits inflammatory response. Thus, it is used for the treatment of cardiac diseases and inflammation. In this study, we aimed to evaluate the effect of an ethanolic extract of SM (SME) on the dental alveolar bone resorption induced by bacterial lipopolysaccharide (LPS) in rats.

*Materials and methods:* An ethanolic extract was prepared from roots of SM. The major constituents of this extract were determined by high-performance liquid chromatography. The activity of the extract was evaluated in a rat model in which the dental alveolar bone resorption was induced by injection of bacterial LPS into the palatal gingiva around the maxillary molar teeth. The effect of SME on the bone resorption was studied by histologic and histomorphometric analysis.

*Results:* The number of osteoclasts and the percentage of osteoclasts covering the alveolar bone surfaces were significantly increased in the LPS group compared with those in the phosphate-buffered saline (PBS) group. The number and percentage of the osteoclasts on the bony surfaces were significantly reduced in the SME group in comparison with the LPS group, although it was still higher than the numbers observed in the PBS group.

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**Conclusion:** Because SME reduced bone resorption caused by the injections of bacterial LPS in rats, we suggest that SME might have a protective effect on dental alveolar bone resorption in periodontitis.

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## Introduction

Periodontitis, a disease of the periodontium characterized by destruction of tooth-supporting tissues, is caused by a host-mediated inflammatory response to bacterial plaque residing in gingival pockets and can eventually lead to tooth loss.<sup>1</sup> Several species of Gram-negative bacteria are known to be key etiologic pathogens of periodontitis.<sup>2</sup> These bacteria, as well as their biological exudates, can induce inflammatory cell infiltration, edema, and vascular dilatation in inflamed tissues.<sup>3</sup> Although the detailed pathogenesis of the disease is still being investigated, significant effort is now focused on finding pharmacological agents that can treat periodontitis by either acting against the causative bacteria or reducing the host inflammatory response.<sup>4</sup>

Dried roots of *Salvia miltiorrhiza* (SM) Bunge (Labiatae/Lamiaceae; common name danshen) are listed in the Chinese Pharmacopoeia as a treatment for cardiovascular disease and inflammation.<sup>5</sup> A water decoction of SM is reported to be effective for improving reperfusion of ischemic myocardium and enhancing blood circulation.<sup>6</sup> Similarly, an ethanolic extract of SM (SME) inhibits the inflammatory response associated with myocardial infarction and atherosclerosis.<sup>7,8</sup> The potential anti-inflammatory effects of SME were evaluated both *in vitro* and *in vivo*. The anti-inflammatory effects of SME on lipopolysaccharide (LPS)-induced nitric oxide production and inducible nitric oxide synthase expression in RAW 264.7 cells were demonstrated previously. In addition, SME was shown to significantly reduce inflammation on carrageenan- or dextran-induced acute arthritis in rats.<sup>9</sup> Results of antimicrobial studies indicated that the major portion of its activity was due to the presence of tanshinones and phenolic acids in its hairy roots.<sup>10</sup> The mechanism of antimicrobial

activity of the hexane fraction of SM Bunge against *Staphylococcus aureus* and methicillin-resistant *S. aureus* was evidenced by its ability to inhibit the expression of the resistant genes, *mecA*, *mecR1*, and *femA*, in messenger RNA.<sup>11</sup> Additional bioactivities of the SM have also been reported, including antineoplastic and antiosteoporotic effects.<sup>12,13</sup> Recently, it has been reported that SME can ameliorate the effect of periodontal damage induced by silk ligation around the tooth neck area in a rat model.<sup>14</sup> We herein evaluated whether the ethanolic SME could inhibit bone resorption in rats with the experimental periodontitis induced by LPS injection; the antimicrobial effect of ethanolic SME was not considered in this study.<sup>14</sup>

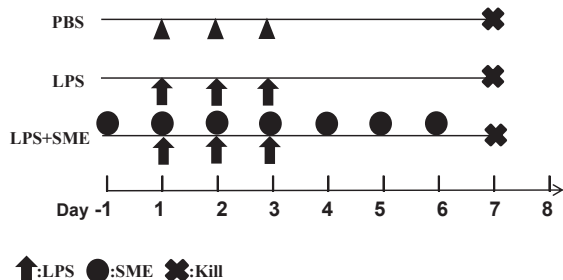
## Materials and methods

### Preparation of the ethanolic extract of SM root

SME was prepared as described previously.<sup>15</sup> In brief, 1 kg of dried SM roots were pulverized and soaked in 10 L of 95% ethanol for 72 hours at room temperature. The extract was filtered and concentrated by evaporation under vacuum. The resulting solid residue was soaked in 95% ethanol (10 L) for 48 hours, filtered, and then concentrated again. This soak-filter-evaporation process was repeated two more times, and the filtrates were evaporated to dryness, yielding 100.3 g of crude SME (10% yield). A quantitative analysis of SME was carried out by reverse-phase high-performance liquid chromatography (Waters XBridge Shield RP18, Hitachi, Tokyo, Japan, 5  $\mu$ m, 4.6 mm  $\times$  250 mm) using a mobile phase of 75% acetonitrile/25% H<sub>2</sub>O at a flow rate of 1.0 mL/min with an injection volume of 10  $\mu$ L and a UV detection wavelength of 254 nm. The extract was then dissolved in 10% Tween 80 for the *in vivo* experiment.

### LPS-induced osteoclast resorption from the dental alveolus of rats

The effect of SME on LPS-induced osteoclast resorption from the dental alveolus was evaluated after administering injections of *Escherichia coli* LPS [dissolved in phosphate-buffered saline (PBS) solution, 5 mg/mL, *E. coli* serotype O55:B5; Sigma Co., St. Louis, MO, USA] into the palatal gingiva of rats as described previously.<sup>16–18</sup> In brief, 15 rats were randomly divided into the following three treatment groups based on the treatment they received: PBS, LPS, and SME treatment groups. Rats in the PBS treatment group received a 10- $\mu$ L PBS injection daily for 3 days; rats in the LPS treatment group received a 10- $\mu$ L LPS (5 mg/mL) injection for 3 days; and rats in the SME group received the same LPS injections for 3 days and a



**Figure 1** Experimental design for the lipopolysaccharide-induced dental alveolar bone resorption in rats. LPS = lipopolysaccharide; PBS = phosphate-buffered saline; SME = *Salvia miltiorrhiza* extract.

daily 300 mg/kg intraperitoneal dose of SME for 7 days, beginning 1 day prior to the 1<sup>st</sup> day of LPS injection. At the end of the 7-day treatment period, all animals in the three groups were killed and palatal specimens were taken and prepared for histologic analysis (Figure 1). In this study, all animals were housed in a dedicated, pathogen-free facility and were handled in accordance with protocols approved by the Institutional Animal Care and Use Committee, National Defense Medical Center, Taipei, Taiwan (IACUC-11-033).

### Histology and histometric analysis

After fixation, the specimens were decalcified with 14% EDTA and embedded in paraffin. The maxillary specimens were then cut into 4- $\mu$ m-thick sections in the bucco-palatal direction. All sections were routinely stained with hematoxylin and eosin (H&E). Tartrate-resistant acid phosphatase (TRAP) staining was further performed on the each section to determine the level of active osteoclasts on the bony surface. The TRAP solution was prepared as follows: 9.6 mg naphthol AS-BI phosphate substrate (Sigma) was dissolved in 0.6 mL *N,N*-dimethylformamide (Sigma) with 60 mL of 0.2M sodium acetate buffer (pH 5.0, Sigma) containing 84 mg of Fast red-violet LB diazonium salt (Sigma), 58.2 mg of tartaric acid (Sigma), and 240  $\mu$ L of 10% MgCl<sub>2</sub>. The mixture was then filtered through a 0.22- $\mu$ m pore-size filter. Slides were incubated for 5 minutes in the staining solution at 37°C in the dark. Afterward, the slides were

washed with water for 30 minutes, and then counterstained with H&E for 6 minutes.<sup>19</sup>

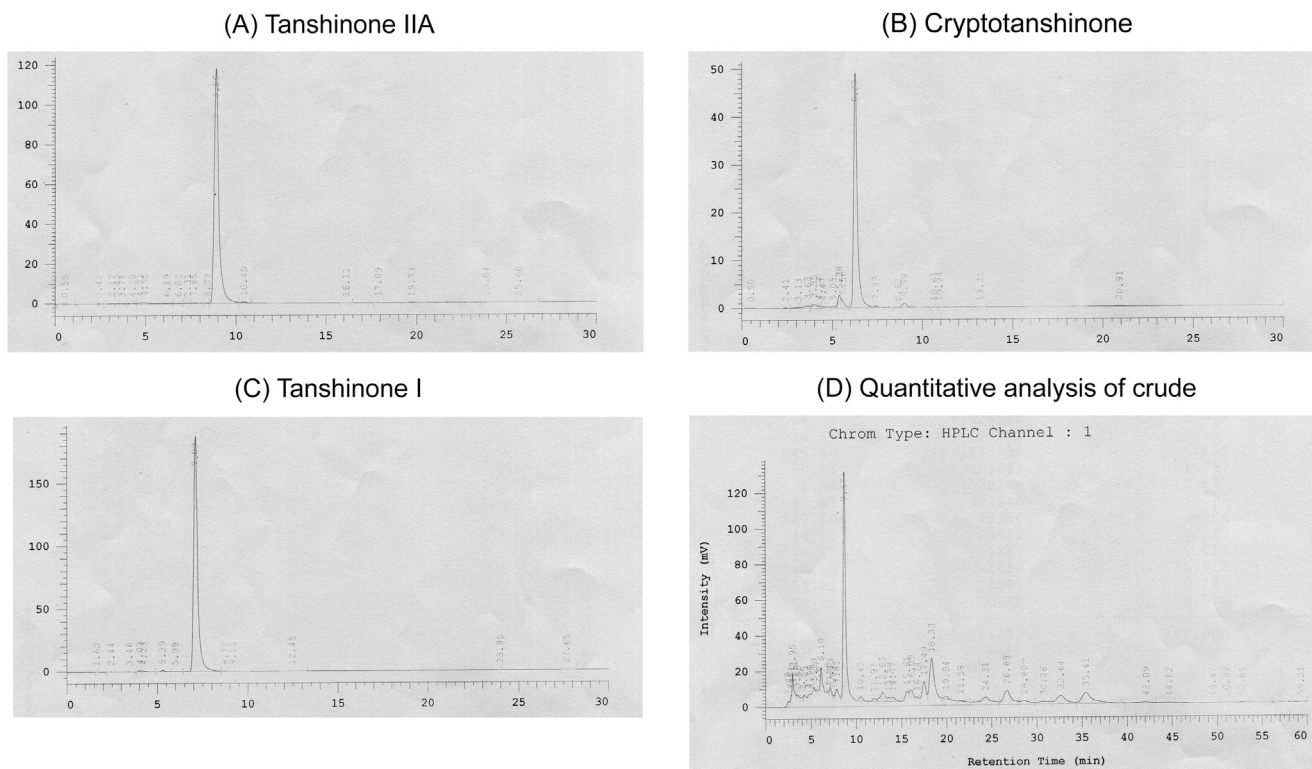
A total of eight slides were selected from the 40 slides of each specimen (three sections on one slide) for histologic and histometric analysis. The number of TRAP-positive osteoclasts with three or more nuclei on the palatal bony lacunae identified in the area between the bone crest and the great palatine vessels were recorded.<sup>16,20</sup> The bone resorption was quantified statistically in terms of the number of osteoclasts (number of osteoclasts/alveolar bone surface, no./mm) and the percentage of the bone surface covered with osteoclasts (osteoclast surface/alveolar bone surface, %).<sup>21–23</sup>

### Statistical analysis

All data were analyzed using a statistical software package (SPSS version 15.0, IBM, Chicago, IL, USA). The effect of SME on the number of osteoclasts measured on surfaces of dental alveolar bones in the three study groups was examined by one-way analysis of variance and analyzed with a Duncan test (*post hoc*). A P value less than 0.05 was considered to indicate a significant difference.

### Results

The major components of 95% ethanolic SME were determined by comparison with authentic standards. In this



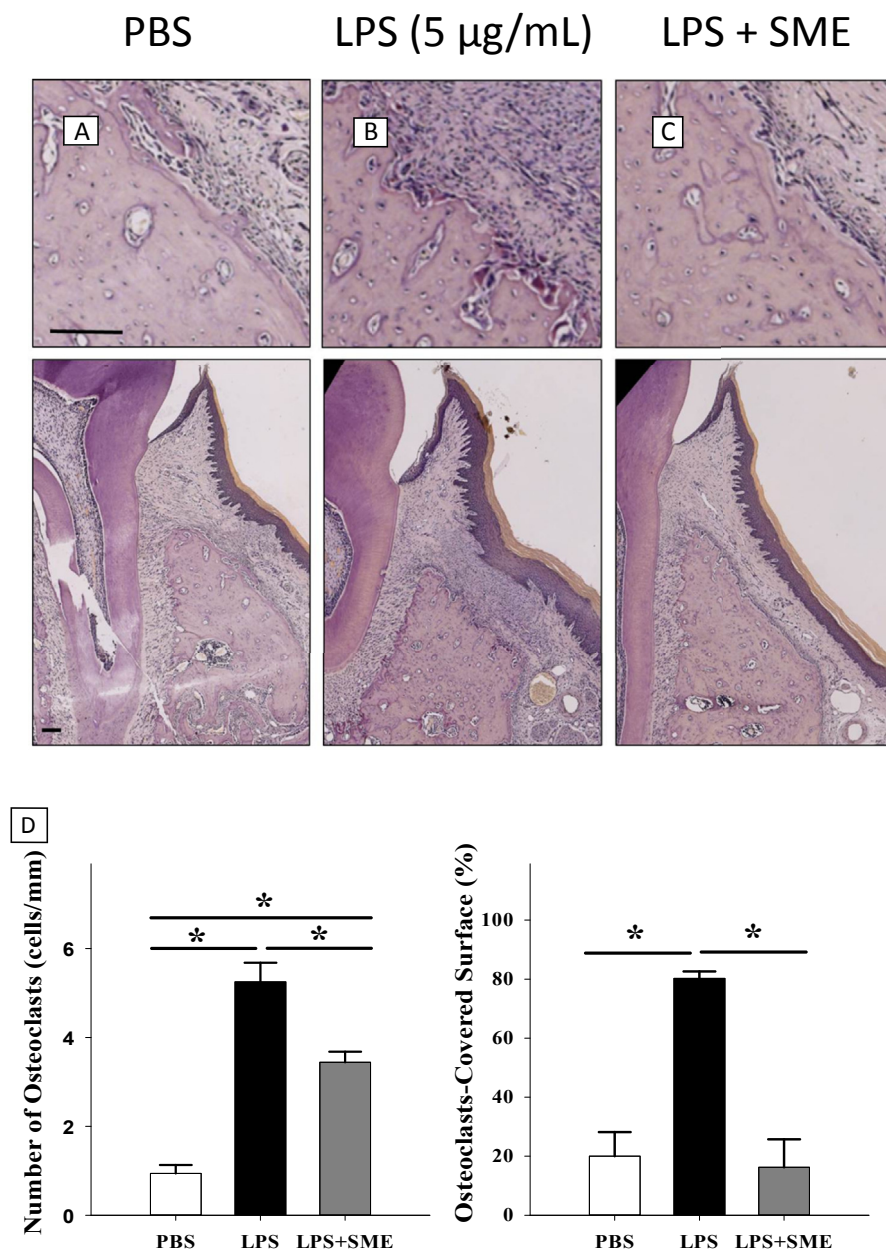
**Figure 2** Quantitative analysis of crude *Salvia miltiorrhiza* extract (SME) and authentic standards for retention time of each substituent. The quantitative analysis of SME was performed by reverse-phase high-performance liquid chromatography with a UV detection wavelength of 254 nm. Upon comparison with authentic standards (A–C), the major SME components were identified as 30% tanshinone IIA, 5.8% cryptotanshinone, and 2.8% tanshinone I (D).

study, the major components were 30% tanshinone IIA, 5.8% cryptotanshinone, and 2.8% tanshinone I (Figure 2).

On the palatal bony surface in specimens from rats in the PBS control group, we observed a small number of osteoblasts with osteoid underneath (Figure 3A). By contrast, many TRAP-positive-stained osteoclasts with resorptive lacunae were observed in specimens from rats in the LPS group (Figure 3B). Importantly, the number of osteoclasts was significantly reduced by SME treatment compared with the LPS group, although it was still higher than that observed in the PBS group (Figures 3C and 3D).

## Discussion

SME, especially tanshinone IIA as an active component of the ethanolic extracts, is a very popular medicinal formulation that has been extensively used to treat various diseases, such as myocardial infarction and atherosclerosis.<sup>7,8</sup> However, in other extraction processes using a ratio of water to raw SM, polysaccharides were reported as the main antioxidant component.<sup>24</sup> In two previous studies, higher amounts of tanshinones were obtained with a higher percentage of ethanol extract, and this component was



**Figure 3** Effect of *Salvia miltiorrhiza* extract (SME) on lipopolysaccharide (LPS)-induced osteoclast resorption on the dental alveolar bony surface. (A–C) The palatal histology of rats in the phosphate-buffered saline (PBS), LPS (5 µg/mL), and LPS + SME groups. Sections are tartrate-resistant acid phosphatase stained and shown at 200× and 40× magnifications in the upper and lower rows, respectively (scale bar = 50 µm). (D) The number of osteoclasts (number of cells/mm) and the percentage of osteoclasts-covered surfaces (%) measured in the three animal groups (\* significant difference at  $P < 0.05$ ).

used as an ingredient in health-care foods to improve the effectiveness of chemotherapeutic agents of cancer<sup>25</sup> and drugs for cardiovascular protection.<sup>26</sup> In our study, about 30% tanshinone IIA, 5.8% cryptotanshinone, and 2.8% tanshinone I were obtained by using 95% ethanol extract, which demonstrated the inhibitory effect of SME on LPS-induced alveolar bone resorption. These data indicate that using the ethanol fraction of SM increases the amounts of tanshinones obtained, which exhibits significant anti-inflammatory and bone resorption inhibition effects.

The SME also inhibited dental alveolar bone resorption induced by the trans-gingival injection of bacterial LPS in our study. Several previous studies have also shown that periodontitis and associated tissue destruction can be induced in animals by dietary manipulation,<sup>27</sup> injection of bacterial toxins,<sup>14</sup> placement of peri-dental silk ligatures or orthodontic elastics for bacterial colonization, or surgical removal of alveolar bone.<sup>28</sup> Each type of induced periodontitis has certain experimental limitations. The ligation-induced periodontitis, for instance, is thought to cause abnormally exaggerated plaque retention and potential trauma to the local gingival tissue. Although LPS-induced periodontitis model is utilized herein, it does not completely replicate the chronic characteristics of the bacteria-associated periodontitis. However, the presence of the foreign body in the ligation model could prevent the attenuation of the inflammatory process after the treatment agent is tested for use.<sup>18</sup> Recently, it has been reported that SME can ameliorate the effect of periodontal damage induced by silk ligation around the tooth neck area in a rat model. To further exclude the antimicrobial activities of SMEs reported in literature,<sup>11</sup> the trans-gingival delivery of bacterial LPS was chosen to be used in this study.

Several species of Gram-negative bacteria, including *Porphyromonas gingivalis*, are known to be the etiologic pathogens of periodontitis.<sup>2,29</sup> However, the LPS of the bacteria is the key inflammatory mediator, which causes a highly unusual host response. Some studies have suggested that *P. gingivalis* LPS has an ability to stimulate cytokine production comparable to that of *E. coli*, whereas others suggest a much lower potency, including a lower activity in stimulating some cytokines [interleukin-1 (IL-1), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and prostaglandin E<sub>2</sub>] but a comparable activity in stimulating others (IL-6, IL-1ra). In addition, LPS from *P. gingivalis* binds lipopolysaccharide-binding protein 10–100 times less efficiently than that from *E. coli*; additionally, *P. gingivalis* LPS does not interact with toll-like receptors in the same manner as *E. coli* LPS does.<sup>30</sup> Nevertheless, the effect of *P. gingivalis*, rather than *E. coli*, on the bone resorption of this model needs further evaluation.

Our result indicated that treatment with SME significantly reduced bone resorption compared with the control group. This observation is consistent with previous studies, in which SME suppressed bone loss caused by ovariectomy.<sup>31,32</sup> SME was also found to stimulate bone formation within a collagen matrix surrounding surgically created parietal bone defects in New Zealand white rabbits.<sup>33</sup> The authors of that study suggested that SME could be useful for bone grafting, especially in cases with a compromised vascular response. These previous reports are consistent

with our finding that SME has an ameliorative effect on dental alveolar bone resorption associated with periodontitis. Although the detailed mechanism for this effect was not explored in this study, other studies have documented the bioactivity of SME toward osteoblasts, osteoclasts, and macrophages. For example, purified tanshinone IIA, a major lipophilic ingredient of SME, inhibits the production of bacterial LPS-induced inflammatory mediators of prostaglandin E<sub>2</sub>, inducible nitric oxide, and cyclooxygenase-2, as well as inflammatory cytokines in osteoblasts and macrophages (e.g., IL-1 $\beta$ , IL-6, and TNF- $\alpha$ ).<sup>34–36</sup> SME has also been shown to inhibit LPS-induced nuclear factor- $\kappa$ B activation in osteoclasts.<sup>37</sup> Finally, several other lipophilic tanshinones are known to inhibit osteoclast differentiation<sup>38</sup> or LPS-induced inflammatory cytokine production.<sup>39</sup> All of these activities suggest that the mechanism by which SME exerts anti-inflammatory and antiresorptive activity could involve repression of inflammatory signaling. In our study, the antiresorptive effect may have partially resulted from components in SME, particularly tanshinones IIA, which constituted 30% of the major SME components; however, this component is lipophilic and has poor oral bioavailability owing to its low water solubility.<sup>40</sup> Therefore, it is necessary to identify hydrophilic substances in SME, so that the extract could be administered orally (per os).

Our results show a statistically significant inhibitory effect of SME on LPS-induced alveolar bone resorption. We therefore suggest that SME merits further evaluation as an inhibitor of alveolar bone loss caused by bacteria-associated periodontal disease.

## Conflicts of interest

The authors have no conflicts of interest relevant to this article.

## Acknowledgments

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