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Journal of the Chinese Medical Association 81 (2018) 141−146

www.jcma-online.com

Original Article

Scutellaria baicalensis ameliorates the destruction of periodontal ligament via inhibition of inflammatory cytokine expression

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Received February 9, 2017; accepted April 25, 2017

Abstract

Background: *Scutellaria baicalensis* (SB) is widely used as a medicinal plant to treat various inflammatory diseases. In the present study, we investigated the effects of SB on periodontitis in ligature-induced experimental rat model.

Methods: Rats were subjected to a ligature placement around the first molar of the mandible to induce periodontitis. 100 mg/kg SB extracts were orally administered for 14 days. The molar tissues were stained with 1% methylene blue. Histopathological changes of the periodontium were observed by hematoxylin and eosin staining. The levels of cytokines were measured in the gingival tissue.

Results: Alveolar bone resorption was statistically lower in the SB group compared to the ligatured group. SB inhibited the mineralization of cementum. In addition, SB reduced the production of IL-1β, 6, -8 and TNF-α cytokine mRNA expression in gingival tissues.

Conclusion: These results suggest that SB showed ameliorative effects in the ligature-induced periodontitis by inhibition of inflammatory cytokine expression.

Keywords: Alveolar bone loss; Cytokines; Inflammation; Periodontitis; *Scutellaria baicalensis*

1. Introduction

Periodontitis is a common inflammatory disease of tooth-supporting tissues including alveolar bone, cementum, periodontal ligament and gingiva.[1] The primary etiology of periodontal breakdown is the plaque accumulation associated with several species of bacteria such as *Porphyromonas gingivalis*, *Bacteroides forsythus* and *Treponema denticola*.[2] Microbes and their products can initiate periodontal inflammation and induce host immune response. In this process of reaction, immune cells and fibroblasts release various inflammatory molecules that activate the effectors of tissue destruction and further lead to formation of periodontal pocket, loss of tooth attachment and resorption of alveolar bone.[3,4]

Treatment of periodontitis mainly relies on mechanical removal of subgingival plaque and prevention of its accumulation.[5] Instrumental debridement, regarded as an efficient therapy for periodontitis, is not always successful in complete elimination of pathogenic bacteria, especially within the furcation area.[6] In addition, antimicrobial agents are commonly prescribed as adjuvants for infection control. However, a number of studies have been reported that antimicrobial therapies have various adverse effects such as nausea, colitis, diarrhea, dizziness and bacterial resistance.[7] Recently, there has been growing interest in natural products as sources of alternative for periodontal therapy.

The root of *Scutellaria baicalensis* Georgi (Labiatae) is a traditional medicinal herb used extensively in Northeast Asia to treat inflammatory diseases including high fever, diarrhea, dysuria and hematuria.[8] In addition, various studies have confirmed that *S. baicalensis* has anti-inflammatory, anti-

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

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https://doi.org/10.1016/j.jcma.2017.04.013

oxidant, anti-diabetic, neuroprotective and anti-tumor activities.[9–13] In particular, anti-periodontitis effects of *S. baicalensis* by inducing T helper 2-type IgG1 levels have been demonstrated.[14] Baicalin, a main component of *S. baicalensis*, was reported to inhibit tissue damage with its inhibitory effect on cyclooxygenase-2 and inducible nitric oxide synthase expressions.[15] However, the effects of *S. baicalensis* water extract (SB) on periodontitis and underlying mechanism via inhibition of inflammatory cytokines have not been fully defined. The aim of the present study was to evaluate whether *S. baicalensis* could recover alveolar bone loss (ABL) and destruction of ligament tissues by its anti-inflammatory effects in ligature-induced periodontitis in rats.

2. Methods

2.1. Preparation of sample

S. baicalensis roots were obtained from Jung-do Herb, Co., Ltd. (Seoul, Korea). 50 g of dried *S. baicalensis* was extracted with 1 L boiling distilled water for 1 h 30 min. The extracts were filtered, concentrated and lyophilized. The dry weight of the *S. baicalensis* was 16.4 g (yield: 32.77% (w/w)). The voucher specimens were deposited at our laboratory. SB was identified by three standards, baicalin, baicalein and wogonin, using an Agilent Series 1100 HPLC system (Palo Alto, CA, USA) with a binary pump, an auto-sampler, a column oven, and a diode array detector (DAD). The Shiseido UG 120 column (250 × 4.6 mm, 5 μm) was tested with a guard column. The analysis was carried out at a flow rate of 1.0 mL/min with the detection wavelength at 280 nm. The HPLC peaks on SB were synchronized with baicalin, baicalein and wogonin (Fig. 1).

2.2. Animals

Sprague–Dawley rats (male, 7 weeks old) obtained from RaonBio, Inc. (Yongin, Korea). The animals were housed for acclimatization for 1 week. All rats were maintained under standard conditions with controlled temperature and humidity (22 ± 1 °C and 50 ± 5%), under 12-h light/dark cycle. The rats were provided with free access to standard rat chow and water. All experimental procedures were examined and approved by the Committee on the Care and Use of Laboratory Animals of Kyung Hee University (KHUASP(SE)-14-029).

2.3. Experimental design

The rats were randomly divided into three groups (n = 7); NOR, LIGA and SB. In NOR group, rats were non-ligatured with vehicle treatment; In LIGA group, rats were ligatured with vehicle treatment; in SB group, rats were ligatured and treated with 100 mg/kg SB. To induce periodontitis, ligature was placed into the proximal space between the first and

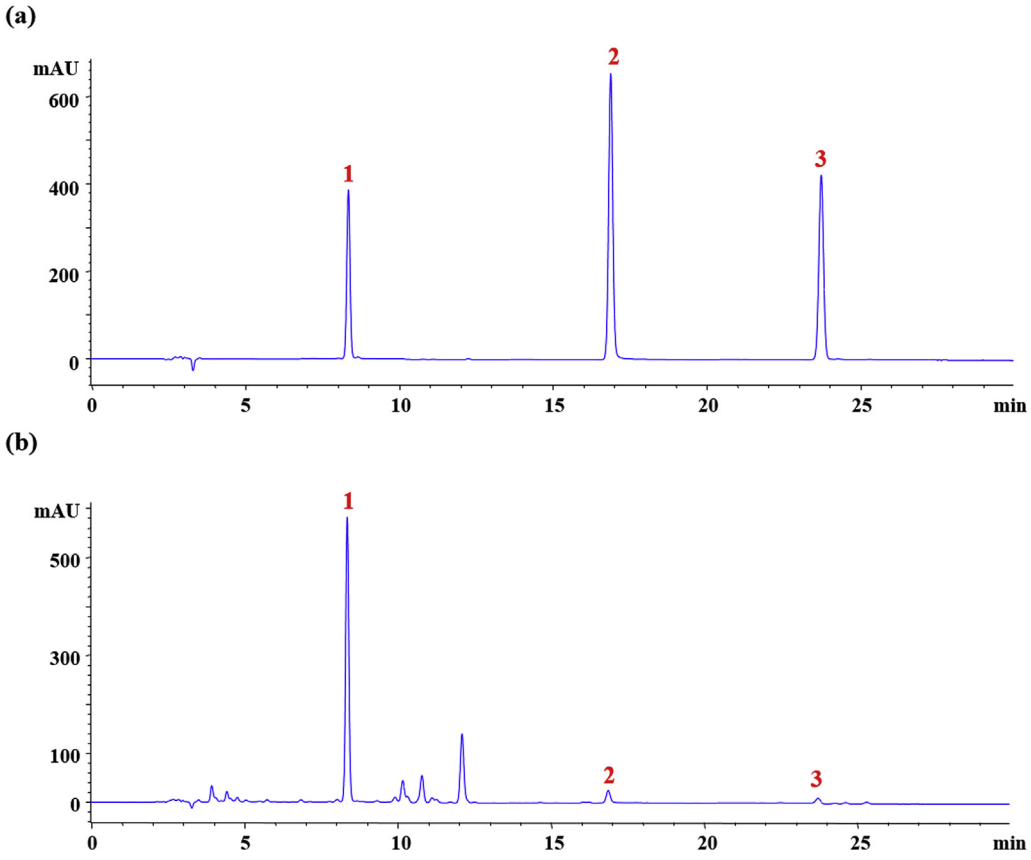

Fig. 1. Identification of SB. HPLC chromatograms of (a) authentic standards (1. Baicalin, 2. Baicalein, 3. Wogonin) and (b) SB.

second molars under anesthesia with intraperitoneal injection of a tiletamine/zolazepam mixture (Zoletil 50; Virbac Lab, Carros, France). This ligature acted as a gingival irritant for 14 days and promoted the accumulation of plaque and subsequent development of periodontal disease.[16] All treatments were started immediately after ligature surgery and continued for 14 consecutive days. 100 mg/kg SB, dissolved in distilled water, was administered by oral gavage. At the end of the experiment, rats were sacrificed. The first mandibular molars on both sides were collected without connective tissues.

2.4. Measurement of alveolar bone loss

The right mandibular molars were stained with 1% methylene blue (Sigma−Aldrich, MO, USA) in distilled water for 5 min. The ABL was assessed morphometrically by measuring the distance between the cement-enamel junction and the alveolar bone crest of the first molars within 3 roots. The molars were photographed using a dermoscope (Smart Microscope Pro; Kangjin Technology, Ltd., Seoul, Korea) with ×10 magnification and were measured with an Image J computerized densitometry system (NIH, Bethesda, MD, USA).

2.5. Histological observation of the periodontium

The left mandibular molar was fixed in 10% buffered formalin (Sigma−Aldrich) for at least 18 h and decalcified in 10% ethylene diamine tetraacetic acid for 2 months at room temperature with general shaking. The decalcified molars were dehydrated with 70%, 80%, 95% and 100% ethanol, and embedded in paraffin. The tissues were sectioned at 7 μm thickness. The deparaffinized mandibular molar sections were stained with hematoxylin and eosin (H&E) for histological changes of the periodontium. All mandibular molar slides were observed using the Leica Application Suite (LAS; Leica Microsystems, Buffalo Grove, IL). The magnification was ×200.

2.6. Evaluation of cytokine production in the gingival tissue

The levels of inflammatory cytokine mRNA expression in the gingival tissue were investigated by reverse transcription polymerase chain reaction (RT-PCR). For RNA extraction, the gingival tissues were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) using a blender, according to the manufacturer's instructions. For complementary DNA (cDNA) synthesis, 1 μg total RNA was mixed with Maxime RT premix (Invitrogen) at 45 °C for 60 min and then at 95 °C for 5 min. To detect the expression of IL-1β, -6, -8 and TNF-α in gingival tissues, cDNAs were then amplified with gene-specific primers using a Maxime PCR premix kit (Invitrogen). Primers used for amplification are listed in Table 1. All PCR products were electrophoresed on 1% agarose gels and determined under UV light after ethidium bromide staining. The relative expression levels of target genes were normalized using GAPDH as an internal control. Visualized bands were quantified using an Image J computerized densitometry system.

Table 1
RT-PCR sequence.

Target gene	Primer	Amino acid sequences	Amplicon size (bp)
IL-1β	5′ Primer	5′-CTC TAG ACC ATG CTA CAG AC-3′	291
	3′ Primer	5′-TGG AAT CCA GGG GAA ACA CTG-3′	
IL-6	5′ Primer	5′- ATC AAC TCC TTC TCC ACA AGC GC -3′	628
	3′ Primer	5′-GAA GAG CCC TCA GGC TGG ACT G -3′	
IL-8	5′ Primer	5′-TGT GGG AGG CTG TGT TTG TA -3′	200
	3′ Primer	5′-ACG AGA CCA GGA GAA ACA GG -3′	
TNF-α	5′ Primer	5′-GGT GCA ATG CAG AGC CTT CC-3′	173
	3′ Primer	5′-CAG TGA TGT AGC GAC AGC CTG G-3′	
GAPDH	5′ Primer	5′-GGC ATG GAC TGT GGT CAT GA -3′	376
	3′ Primer	5′-TTC ACC ACC ATG GAG AAG GC-3	

2.7. Statistical analysis

Significance was determined by one-way analysis of variance (ANOVA) and Dunnett's multiple comparison tests. In all analyses, $p < 0.05$ was taken to indicate statistical significance.

3. Results

3.1. Quality evaluation of SB

Baicalin, baicalein and wogonin, were used as standard compounds for quality evaluation of SB. The peaks on SB were synchronized with baicalin, baicalein and wogonin in HPLC (Fig. 1).

3.2. Effects of SB on alveolar bone loss

A significant difference in the ABL between non-ligatured (NOR: 1061.0 ± 164.9 μm) and ligatured (LIGA: 2187.3 ± 169.5 μm) molar was observed ($p < 0.001$). The level of ABL of 100 mg/kg SB-treated molar was 1826 ± 244.4 μm, significantly lower than that of the LIGA group ($p < 0.05$) (Fig. 2).

3.3. Effects of SB on histological changes of the periodontium

In the NOR group, cementum fully covered pulpal dentin. Several resorption pits were clearly observed lining the surface of dentin in ligature-induced periodontitis. Oral administration

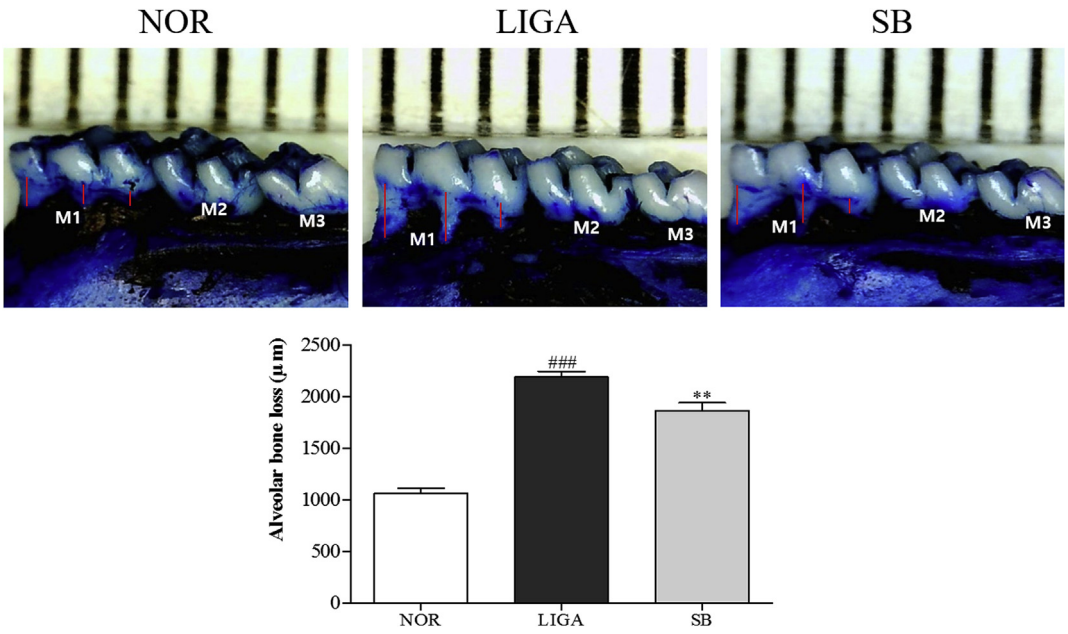

Fig. 2. Representative images stained with methylene blue and quantification of alveolar bone loss in first molar measured by the distance from the cement-enamel junction to the alveolar bone crest (red line). M1 indicates first molar, M2 indicates second molar and M3 indicates third molar. Data from three independent experiments (n = 7) are expressed as means ± S.E.M ($^{###}p < 0.001$; compared with the NOR group, $^{**}p < 0.01$; compared with the LIGA group).

with 100 mg/kg SB recovered the destruction of cementum (Fig. 3).

3.4. Effects of SB on cytokine production in the gingival tissue

The mRNA levels of IL-1β, -6, -8 and TNF-α in LIGA group were ~3.8-fold, ~2.43-fold, ~2.96-fold and ~2.83-fold (respectively) higher compared with the NOR group. These levels were dramatically lower in the SB group. The inhibitory rates of SB on cytokine expression were as follows: 51.0% for IL-1β, 44.1% for IL-6, 57.1% for IL-8 and 48.8% for TNF-α, respectively (Fig. 4).

4. Discussion

Inflammation of gingival sulcus is a hallmark of periodontitis.[17] Release of various inflammatory cytokines resulted from host immune response has been reported to aggravate gingivitis and alveolar bone resorption.[18] Since alveolar bone is one of the important structures supporting jaw bone tissue, continuous destruction of alveolar bone leads to loss of tooth.[19] In this study, severe alveolar bone loss was observed in rats with ligature-induced periodontitis. SB treatment ameliorated the alveolar bone resorption. In addition, resorption pits lining the cementum of alveolar bone were reduced in SB-treated periodontium. These results indicate that administration of SB recovered the destruction of periodontium.

A number of cytokines, followed by inflammation of gingival fibroblast are responsible for development of periodontitis.[19] In particular, IL-1β is a typical cytokine to initiate inflammatory response under periodontal infection.[20] IL-6 has been reported to induce osteoclastogenesis, which leads to alveolar bone resorption, in response to periodontal pathogens.[21] IL-8 plays major roles in trafficking of neutrophils in periodontium. Additionally, TNF-α stimulates matrix metalloproteinase expression, which results in collagen degradation in gingival tissues.[22] These inflammatory cytokines play a major role of destruction of the connective ligament by interaction with periodontal pathogens.[23] It is well known that

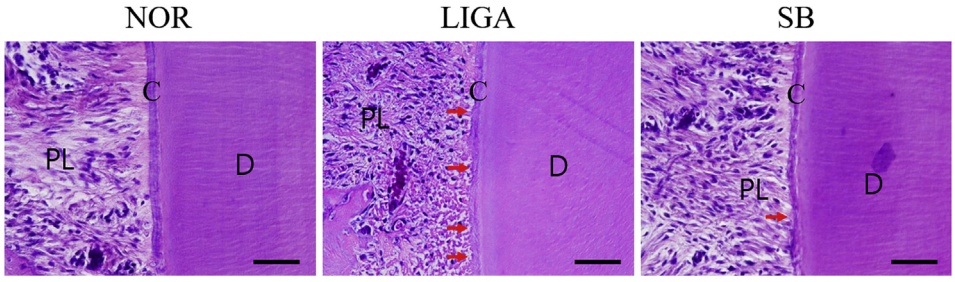

Fig. 3. Representative images stained with H&E under microscope. The red arrows indicate bone resorption pits. PL: periodontal ligament; C: cementum; D: dentin. The magnification was ×200. The scale bar is 100 μm.

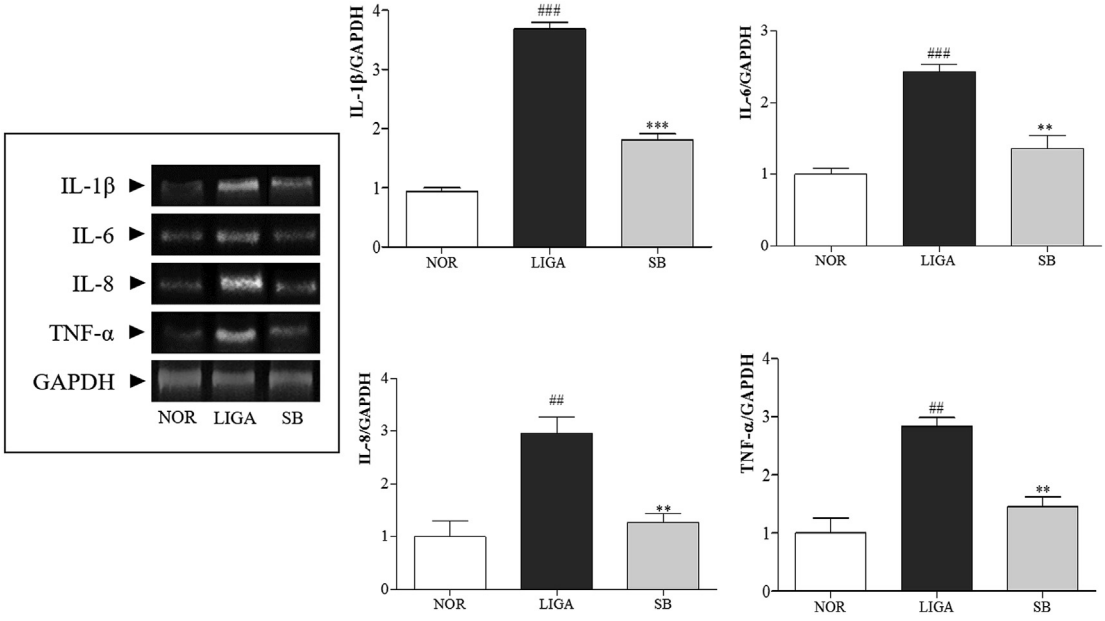

Fig. 4. Representative images of cytokine mRNA expression in gingival tissue and quantification of relative intensity determined by reverse transcription polymerase chain reaction. Data from three independent experiments (n = 7) are expressed as means ± S.E.M ($^{###}p < 0.001$ and $^{##}p < 0.01$; compared with the NOR group, $^{***}p < 0.001$ and $^{**}p < 0.01$; compared with the LIGA group).

SB has been used as an anti-inflammatory agent for treating several diseases. In this regard, we expected the effects of SB on alveolar bone loss could be mediated by inhibition of inflammatory cytokines. SB treatment decreased the elevated expressions of inflammatory cytokines including IL-1β, -6, -8 and TNF-α in periodontitis-affected inflamed gingival tissues. These results reveal that SB had anti-inflammatory effects on ligature-induced periodontitis.

In conclusion, SB inhibited alveolar bone loss, recovered periodontal structures and reduced the expression of inflammatory cytokines. SB ameliorated periodontitis by inhibiting the mRNA expression of inflammatory cytokines in gingival tissues. SB may be a candidate for alternative treatment of periodontitis.

Acknowledgments

This work was supported by a National Research Foundation of Korea Grant funded by the Korean government (NRF-2016R1D1A2B03935368).

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