The effect of *Scutellaria baicalensis* Georgi on immune response in mouse model of experimental periodontitis

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**KEYWORDS**

Experimental periodontitis; immune response; mice; *Scutellaria baicalensis* Georgi

**Abstract** Background/purpose: A medicinal herb *Scutellaria baicalensis* Georgi has been reported to possess antibacterial and anti-inflammatory activities. In this study, the effect of aqueous extracts obtained from *S. baicalensis* GEORGI (*SbE*) on the immune response in ligature-induced periodontitis of mouse models was investigated.

**Materials and methods:** Sixty mice were randomly divided into four groups. Experimental periodontitis was induced by wrapping *Porphyromonas gingivalis*-soaked silk ligature at the gingival margins of the left first maxillary molar, and some of them were treated with *SbE* from the 4th week after the ligature. There were also normal and *SbE*-treated controls without ligature. The histopathologic analyses of periodontia were performed at Weeks 4, 6, 8, and 10 after the ligature. There were also normal and *SbE*-treated controls without ligature. The histopathologic analyses of periodontia were performed at Weeks 4, 6, 8, and 10 after the ligature. Serum levels of immunoglobulin (Ig) G isotypes, e.g., IgG1 and IgG2a, were quantified by enzyme-linked immunosorbent assay.

**Results:** Compared with the experimental periodontitis group, the periodontitis + *SbE*-treated mice showed significantly improved histopathology of periodontal tissue at Weeks 6, 8, and 10 after the ligature (*P* < 0.05). Compared with the control groups, periodontitis + *SbE*-treated mice exhibited significant higher levels of Th2-type IgG1 at Weeks 6, 8, and 10 (*P* < 0.01), whereas levels of the Th1-type IgG2a were relatively unaffected in periodontitis + *SbE*-treatment group but significantly increased in experimental periodontitis group at Weeks 6, 8, and 10 (*P* < 0.01).

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Introduction

Periodontal diseases are chronic infectious inflammatory diseases characterized by the destruction of tooth-supporting structures. The interplay between periodontal pathogens and the host immune system is responsible for the pathological process in chronic periodontitis. Host inflammatory mediators have been associated with tissue destruction, while anti-inflammatory mediators counteract and attenuate disease progression. Recent studies have demonstrated that cytokines [tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ)], considered harmful in the context of tissue destruction, play important roles in the control of periodontal infection. Studies by mouse models have also demonstrated that experimental periodontitis is a Th1-type immune response. However, it has been well established that the balance of Th1/Th2 can affect the outcome of many diseases. With the discovery of several T-cell subsets bearing distinct immunoregulatory properties, and both Th1 and Th2 cytokines play an important role in maintaining alveolar bone homeostasis in chronic periodontitis. The adoptive transfer experiments have consistently shown an active role for Th1 cytokines in periodontal disease exacerbators and Th2 cytokines as periodontal disease protectors. Studies showed that Th1 inflammatory mediator levels quantitatively dominated over the Th2 mediators in chronic periodontitis. The levels of interleukin (IL)-4 and IL-10 in gingival crevicular fluid (GCF) in patients with chronic periodontitis are significantly lower compared to the healthy controls, and periodontitis is prevented or ameliorated by treatment with IL-10. In addition, the increased ratio of IL-4 to IFN-γ is also related to the improvement of clinical periodontal health. After periodontal therapy, a trend for decreased Th1 mediator levels and increased Th2 levels has been observed.

Porphyromonas gingivalis has been considered as one of the important periodontal pathogens. Immune responses against P. gingivalis play a crucial role on the pathogenesis of periodontal disease. The humoral immune response against P. gingivalis in patients with chronic periodontitis has been extensively assessed, and a direct relationship between the serum anti-P. gingivalis immunoglobulin (Ig) G levels and subgingival P. gingivalis colonization has been proved in many studies. Patients with chronic periodontitis have a significantly higher IgG level than periodontally healthy controls. Analysis of the IgG subclass responses revealed that there is a corresponding increase in the specific IgG2 response as mean probing depth increased, while a high serum IgG2 response is associated with a predominantly Th1 response. When the RgpA-Kgp proteinase adhesin complex is used as a parenteral vaccine in the mouse periodontitis model, it induces a Th2 response (e.g., a high-titer IgG1 Ab response) that blocks function of the RgpA-Kgp complex and protect against periodontal bone loss, which demonstrated that predominantly Th2 response is responsible for the protection of periodontitis.

The roots of the traditional Chinese herbal medicine Scutellaria baicalensis Georgi have been widely employed for many centuries as a popular antibacterial, antiviral, and anti-inflammatory agent, and widely used in the treatment of a variety of inflammatory diseases such as bronchitis, nephritis, hepatitis, and asthma. Thus far, there are only a few studies that have investigated the effects of baicalin on the periodontal disease, mainly focused on its pharmacological activities as an anti-inflammatory and antioxidant agent. The effect of S. baicalensis Georgi on immune regulation in periodontitis has not been reported. Therefore, our study was designed to determine whether the extracts of S. baicalensis Georgi could influence the progression of experimental periodontitis by inducing a protective immune response in ligature-induced periodontitis in mice.

Materials and methods

Preparation of the aqueous extractions of herbal medicine

S. baicalensis Georgi was purchased from Guangzhou Zhixin Pharmaceutical Co., Ltd (Guangzhou, China). The aqueous extracts of S. baicalensis (SBE) were prepared by a previously reported method. Briefly, 100 g of sliced roots of S. baicalensis was soaked in 1000 mL of double distilled water for 2 hours, and then was boiled under reflux at 95°C for 1.5 hours for the first extraction step; the above process was repeated for the second extraction. After reflux, the samples were filtered and evaporated using a rotary evaporator and lyophilized to yield extract powder.

P. gingivalis-adhered ligatures

P. gingivalis ATCC 33277 was cultured and maintained in modified chopped meat medium supplemented with 5 μg/mL hemin (Sigma-Aldrich, Shanghai, China) and 0.5 μg/mL menadione (Sigma-Aldrich) at 37°C in an anaerobic environment consisting of 90% N₂, 5% CO₂, and 5% H₂. For periodontal infection, P. gingivalis-adhered silk ligatures were prepared. In brief, a 10-mm-long 5-0 sterile silk ligature was immersed in brain–heart infusion broth supplemented with 5 μg/mL hemin and 0.5 μg/mL menadione. P. gingivalis was then inoculated and cultured at 37°C in an anaerobic environment for 72 hours.
Animal models

Kunming mice (12 weeks old, male) that were specific pathogen-free (SPF) grade were purchased from the Experimental Animal Center of Sun Yat-sen University (Guangzhou, China). A total of 60 mice were included in this study. The mice were divided into four groups, consisting of 12 mice for the uninfected controls and 16 mice for the other groups. The mice were maintained in a pathogen-free environment and housed in air conditioning at above 22°C with standard 12-hour light/12-hour dark cycles. Periodontitis was induced by wrapping *P. gingivalis*-soaked 5-0 silk ligatures at the gingival margins of the left first maxillary molar and changing the ligatures every week. The experimental periodontitis group was gavaged with distilled water (0.2 mL/day) from the 4th week after the ligature; SbE-treatment group were gavaged with SbE (50 mg/kg/day by oral gavage, once a day) from the 4th week after the ligature. The uninfected control group without ligature was gavaged with distilled water (0.2 mL/day) and the SbE-treated control group without ligature was gavaged with SbE (50 mg/kg/day) from the 4th week. Mice were monitored daily and were sacrificed at Weeks 4, 6, 8, and 10 after the ligature, and three to four mice per group at each time point were sacrificed. All the experiments were performed in compliance with the requirements of the Animal Ethics Committee at Jinan University (Guangzhou, China).

Histological observations

The mice were sacrificed by ether inhalation at Weeks 4, 6, 8, and 10. Immediately after sacrifice, the maxillae were removed and fixed in 10% buffered formalin for a period over 48 hours. Specimens were trimmed. Only molar and periodontal tissues were retained. Specimens were then decalcified and processed for histology using standard methods. Combined tooth and periodontal tissue slices were performed in a microtome in the buccal-lingual direction, 5-μm tissue sections (50- or 100-μm distance between the sections) of the tissue from each mouse were cut, dewaxed, rehydrated, and stained with hematoxylin and eosin (H&E) (Sigma-Aldrich, Shanghai, China). The inflammatory changes on each section were evaluated under light microscopy from at least three sections per animal. The grade of necroinflammation in the periodontal tissues semiquantitatively was assessed on a scale from 0 (normal) to 3 according to the method of Huang et al as follows: 0, normal histology; 1, mild inflammation without necrosis; 2, moderate inflammation without necrosis; and 3, severe inflammation with necrosis.

Measurement of anti-*P. gingivalis* IgG isotypes in serum

Mouse blood was collected from the retro-orbital sinus. Serum was prepared by centrifugation at 2500 rpm for 10 minutes and stored at −20°C until use. Serum levels of IgG1 and IgG2a were quantified by enzyme-linked immunosorbent assay (ELISA) from individual animals. Ninety-six-well microtiter plates (Costar, Corning, NY, USA) were coated with 10 μg/mL protein of heat-killed *P. gingivalis* and incubated overnight at 4°C. After washing with 0.05% Tween in PBS and blocking with 2% BSA at 37°C, serial dilutions of sera were added and incubated for 2 hours at 37°C. Plates were washed and added with a peroxidase-conjugated rabbit antimouse IgG1 and IgG2a (1/4,000; Southern Biotech, Birmingham, Al., USA) for 1 hour at 37°C. Tetramethyl benzidine was used as the substrate (Sigma-Aldrich). The reaction was stopped 30 minutes later by the addition of 2M H2SO4. The optical density (OD) values were measured at 450 nm with an automatic microplate reader (Model 550; Bio-Rad).

Data analysis

Results of experimental studies are reported as mean ± standard deviation, and differences were analyzed by using the Student t test or the Wilcoxon signed-rank test. A value of P < 0.05 was considered statistically significant.

Results

Histological observations

Histologically, there were no changes in junctional epithelium, periodontal ligament fibers, and the morphology of alveolar bone in the normal controls (Fig. 1A) and SbE-treated controls without ligature (Fig. 1B). A severe periodontal inflammation was observed at Week 6 in the experimental periodontitis group (Fig. 1C), and an even more severe periodontal inflammation and severe alveolar bone resorption was observed at Week 8 (Fig. 1E). However, only moderate inflammation was observed in the periodontal tissue at Week 6 (Fig. 1D) and moderate inflammation was observed in the periodontal tissue and fibroblasts with capillary hyperplasia in the periodontal tissue at Week 8 (Fig. 1F) in the periodontitis + SbE-treatment group. Severe periodontal inflammation and alveolar bone resorption was observed in the experimental periodontitis group (Fig. 1G) and moderate inflammation in periodontitis + SbE-treatment group (Fig. 1H) in the periodontal tissue at Week 10. Semi-quantitative analysis of the severity of inflammation and necrosis of the periodontal tissues of different groups is shown in Table 1. Compared with the periodontitis group (n = 6), there was significantly decreased inflammation in the periodontal tissues from periodontitis + SbE-treatment (n = 6) at Weeks 6, 8, and 10 after the ligature (P = 0.027, 0.026, and 0.026, respectively).

Levels of IgG isotypes in serum

IgG1 and IgG2a isotypes of immunoglobulins are surrogates of Th2 and Th1 phenotypes of immune responses, respectively. The levels of IgG1 and IgG2a in mouse serum in different groups at different times are shown in Figs. 2 and 3, respectively. Compared with normal or SbE-treated controls without ligature, the levels of IgG1 were not significantly affected in the experimental periodontitis group at all times (P > 0.05). However, the levels of IgG1 in...
periodontitis + SbE-treatment group were significantly higher than those in the periodontitis group at Weeks 6, 8, and 10 (P < 0.01) (Fig. 2). Compared with normal or SbE-treated controls without ligature, the levels of IgG2a concentration in serum were significantly increased in the periodontitis group at all times (P < 0.01), and in the periodontitis + SbE-treatment group at Weeks 4, 6, and 8 (P < 0.01). However, the levels of IgG2a in the

Figure 1  Histological assessment of periodontal destruction. (A) Periodontal tissue from an uninfected control. (B) Periodontal tissue from a SbE-treated control without ligature; no inflammation was found. (C) Periodontal tissue from a mouse of the experimental periodontitis group; severe inflammation is observed at Week 6. (D) Periodontitis + SbE-treated group at Week 6, moderate inflammation is observed in the periodontal tissue. (E) Periodontal tissue from a mouse from the periodontitis group, which developed severe inflammation and bone resorption at Week 8. (F) Periodontitis + SbE-treated group at Week 8; moderate inflammation is observed in the periodontal tissue and fibroblasts are observed with capillary hyperplasia. (G) The experimental periodontitis group at Week 10; severe periodontal inflammation and alveolar bone resorption are observed in the periodontal tissue. (H) Periodontitis + SbE-treated group at Week 10, and moderate inflammation is observed (H&E staining; original magnification ×40). H&E = hematoxylin and eosin; SbE = Scutellaria baicalensis Georgi.
periodontitis + SbE-treatment group were significantly lower than those in the periodontitis group at Weeks 6, 8, and 10 (P < 0.01) (Fig. 3).

Discussion

Treatments of periodontitis and prevention protocols for individuals at risk are based on mechanical debridement of infectious sites and pharmacological antibacterial therapy. These treatments usually involved frequent office visits over a long period of time and are costly to both the patient and society. New and more effective approaches to the prevention and control of the disease are needed. The existing evidence from human and animal studies suggests that immunization to P. gingivalis may be beneficial in controlling and preventing periodontitis. However, there is contradiction between protective immune response represented by high antibody titers and disease progression. Houri-Haddad et al.24 reported that immunization to P. gingivalis induces a protective as well as destructive response. It has been reported that the traditional Chinese medicinal herb, an herbal extract obtained from S. baikalensis Georgi, has fewer side effects in animal model.24 Baicalin, a flavonoid compound isolated from S. baikalensis, has been shown to possess a number of pharmacological effects including antiviral, anti-inflammatory, antioxidant and immune regulation, and stimulating human T and B cells proliferation.27 Therefore, the present study investigated the effect of the extract of S. baikalensis Georgi on Th1/Th2 responses in murine ligature-induced periodontitis by determining the IgG subclasses, and our data suggested that the aqueous extracts obtained from S. baikalensis Georgi (SbE) could induce Th2 type IgG1 response in ligature-induced periodontitis in mouse models.

Periodontal disease and airway allergic inflammation present opposing inflammatory immunological features and clinically present an inverse correlation.28 However, anti-inflammatory Th2- and Treg-related cytokines such as IL-4 and IL-10 exert the reverse effect, being associated with lower periodontal disease severity.29–31 Studies have associated Th1/Th2 type of response with the expression of IgG subclasses. Th1 cytokines induce the production of the IgG2a subclass and Th2 derived cytokines support the IgG1 subclass.32 Houri-Haddad et al.32 observed higher IgG1 expression in an alum-based adjuvant while examining the effect of immunization to P. gingivalis on inflammatory response in mice and concluded that the use of an alum-based adjuvant shifts the T-cell response towards a Th2-dominance response. Goncalves et al.33 demonstrated that infection with P. gingivalis results in a Th1 response with high titers of IgG2a isotype antibodies in mice. Here, our study demonstrated that the experimental periodontitis induced by infection of P. gingivalis stimulated a proinflammatory, Th1 type IgG2a, while periodontitis + SbE-treatment stimulated higher levels of Th2 type IgG1 to P. gingivalis and shifted the immune response towards an anti-inflammatory, Th2-dominant response. Studies have reported higher antibody titers of IgG to P. gingivalis in sera from adult periodontitis patients than that in sera from healthy subjects.34–36 Analysis of the IgG subclass reveals correlation of the IgG2 responses with more severe disease and indicates that a high serum IgG2 response may be associated with a predominantly Th1 response.17 The immunoglobulin class switch to generate IgG2a is solely dependent on IFN-γ, and a high-titer IgG2a immune response has been generally accepted as a strong surrogate of a typical Th1 response.37 In addition, S. baikalensis Georgi has strong anti-inflammatory properties by inhibition of proinflammatory Th1 cytokine IL-1β, IL-2, IL-6, IL-12, and TNF-α expression.38 Chung et al.39 found that the extract of S. baikalensis Georgi expresses a significant inhibitory effect

![Figure 2](image-url) The serum IgG1 levels. OD values are means from triplicate measurements and data are presented as means ± SEM. Four mice are used per group and the results shown are representative of two independent experiments. * Significantly different from the value for control mice (P < 0.01). OD = optical density; PD = periodontitis; SbE = Scutellaria baikalensis Georgi; SEM = standard error of the mean.

### Table 1 Comparison of inflammatory scores in the periodontal tissues of different groups at different times.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Inflammatory scores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 wk</td>
</tr>
<tr>
<td>Normal controls</td>
<td>0, 0, 0</td>
</tr>
<tr>
<td>SbE-treated controls</td>
<td>0, 0, 0</td>
</tr>
<tr>
<td>Periodontitis</td>
<td>1.5, 1.6, 1.4, 1.5, 1.6, 2.4, 2.4, 2.3, 2.2, 2.3, 2.9, 2.8, 2.9, 2.8, 2.9, 2.8, 2.5, 2.6, 2.5, 3, 2.5</td>
</tr>
<tr>
<td>Periodontitis + SbE-treatment</td>
<td>1.4, 1.5, 1.4, 1.5, 1.6, 2.0, 1.8, 1.9, 1.9, 1.8, 2.0, 1.9, 1.9, 2.0, 1.9, 1.8, 1.5, 1.4, 1.3, 1.5, 1.5*</td>
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*P < 0.05 vs. periodontitis group.

SbE = Scutellaria baikalensis Georgi.
in LPS-induced production of IL-1β. Krakauer et al. reported that baicalin inhibits antigen-induced inflammatory cytokines and chemokines. Therefore, increased IgG1 suggests that treatment with the extracts of S. baikalis Georgi may shift the T-cell response away from a Th1-dominance response toward Th2-dominant response. IgG1 is representative of a Th2 cytokine response. The increased levels of IgG1 in periodontitis + SbE-treated group suggest that the extracts of S. baikalis Georgi may also be associated with the up-regulation of anti-inflammatory (Th2) response.

S. baikalis Georgi has been extensively used in China and Japan as an anti-inflammatory and anti-infectious agent for many centuries, without a significant detrimental effect. In the current study, our data showed that compared with the experimental periodontitis group, the periodontitis + SbE-treated group showed significant milder histopathology of periodontitis at Weeks 6, 8, and 10. It has been reported that baicalin, one of the flavonoids from the dried root of S. baikalis, protects against tissue damage in ligature-induced periodontitis in rats, in which baicalin-treated rats showed significantly lower alveolar bone loss and significantly higher density of collagen fibers than those in the experimental periodontitis group; which may be mediated by its inhibitory effect on the expression of cyclooxygenase-2 and inducible nitric oxide synthase. Moreover, baicalin may inhibit cyclooxygenase-2 and inducible nitric oxide synthase expression, reducing the overproduction of nitric oxide and prostaglandin E2, and thus reduces bone resorption. Our data suggested that the anti-inflammatory effect of aqueous extracts of S. baikalis Georgi on experimental periodontitis in mouse model was probably due to its Th2-dominance response. Further studies are needed to investigate the mechanisms of S. baikalis Georgi on the immune response in periodontal disease.

References


