Green tea epigallocatechin-3-gallate alleviates *Porphyromonas gingivalis*-induced periodontitis in mice

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ARTICLE INFO

ABSTRACT

Porphyromonas gingivalis causes inflammation, and leads to the periodontitis in gingival tissue damage and bone resorption. Epigallocatechin-3-gallate (EGCG) is a major polyphenol extract from green tea with plenty of pharmacological functions. The aim of this study was to determine whether continuous oral intake of EGCG would alleviate *P. gingivalis*-induced periodontitis. Eight-week BALB/c mice were administered with EGCG (0.02%) or vehicle in drinking water. They were fed normal food and orally infected with *P. gingivalis* every 2 days, up to a total of 20 times, and then sacrificed at 15 weeks of age. The *P. gingivalis*-challenged group markedly increased alveolar bone resorption of the maxillae in BALB/c mice by Micro-CT detection, and administration of EGCG resulted in a significant reduction in bone loss. Inflammation cytokine antibody array and enzyme linked immunosorbent assay revealed that some inflammatory mediators in serum were increased by *P. gingivalis* infection, but were lowered after EGCG treatment. High positive areas of IL-17 and IL-1β in the gingival tissue were observed in the *P. gingivalis*-challenged mice, and were reduced by EGCG treatment. Real-time polymerase chain reaction (PCR) analyses also showed the expressions of IL-1β, IL-6, IL-17, IL-23, TNF-α and other mediators in gingival tissue were higher in *P. gingivalis*-challenged mice, and were down-regulated with EGCG treatment, except IL-23. Our results suggest that EGCG, as a natural healthy substance, probably alleviates *P. gingivalis*-induced periodontitis by anti-inflammatory effect.

1. Introduction

Periodontitis is a common oral disease and public health problem worldwide. It is a chronic disease resulting in the destruction of tooth supporting tissues, including the resorption of alveolar bone and periodontal ligament, eventually leading to tooth loss and relating to some systemic diseases [1]. Many studies have indicated that chronic inflammatory and immune responses, activated by periodontal infectious pathogens cause damage from gingival tissue to alveolar bone, and can play an important role in the development of periodontitis [2,3].

Porphyromonas gingivalis, a Gram-negative, non-motile, obligate anaerobic bacterium, is one of the most important pathogens associated with chronic periodontitis [4]. The periodontal pathogen infection involves the initial of inflammatory response, and then the host response is amplified by related immune cells in periodontal tissue, as well as inducing bone resorption correlating with inflammatory mediators such as cytokines, chemokines and adhesion molecules [5]. The pathogenicity of *P. gingivalis* including various virulence factors, such

as lipopolysaccharide (LPS), fimbriae, capsule, hemagglutinins and gingipains can induce inflammatory responses in periodontal tissue and alveolar bone resorption [6]. *P. gingivalis* infection not only causes the inflammation in periodontitis, but also relates to systemic diseases, such as cardiovascular disease [7]. Therefore, the prevention of periodontitis caused by *P. gingivalis* is important in both oral and systemic health. Although there are many clinical treatments for periodontal therapy, novel biocompatible natural substances are of interest for prevention and subservience for periodontal treatment.

Green tea is one of the most popular beverages worldwide. Catechin is a natural substance extracted from green tea, and the most abundant catechin is Epigallocatechin-3-gallate (EGCG), which is considered to have protective effects against diabetes, hypertension, cancer and cardiovascular diseases [8]. EGCG has been shown to possess a variety of pharmacological functions, such as anti-oxidative [9], anti-mutagenic [10], anti-angiogenic [11], anti-inflammatory [12] and anti-bacterial [13] effects. We have demonstrated that EGCG attenuates *P. gingivalis*-induced atherosclerosis, owing to its biocompatible properties for anti-inflammatory and anti-oxidative effects [14]. Previous studies have shown that the ratio of *P. gingivalis* was higher than other oral bacteria in chronic periodontitis, and that it invaded periodontal tissue to increase the expressions of inflammatory mediators [15,16], especially interleukins (IL)-1β, IL-6, IL-17 and tumor necrosis factor (TNF)-α.

Several studies have also shown that IL-6 and TNF-α are associated with periodontitis in vivo and in vitro, and are down-regulated by EGCG treatment. However, few studies have reported that EGCG mediates IL-17and IL-1β, which are responsible for receptor activator for nuclear factor-κ B ligand (RANKL) expression and host immune response in periodontal disease during *P. gingivalis* infection. Therefore, the purpose of this study was to investigate whether continuous oral intake of EGCG alleviates the development of periodontitis induced by *P. gingivalis* infection in BALB/c mice, and to demonstrate the anti-inflammatory activities of EGCG by inhibiting inflammatory cytokines expression, including IL-17and IL-1β, as a potential therapeutics to prevent periodontal disease.

2. Materials and methods

2.1. Bacterial strain

P. gingivalis strain FDC381 was cultured on anaerobic basal agar plates (Oxoid LTD, England) enriched with 5% sheep blood under anaerobic condition with 80% N_2, 10% CO_2 and 10% H_2 at 37 °C for 3–5 days. Cultures were then inoculated into brain heart infusion broth (Oxoid LTD, England), supplemented with 5 μg/mL hemin and 0.4 μg/mL menadione (Sigma-Aldrich, USA), and grown for 2 days until it reached an optical density at 600 nm (OD_{600}) of 1.0, corresponding to 10^9 CFU/mL. The cultured cells were then centrifuged at 8000 g for 20 min at 4 °C and diluted by phosphate-buffered saline (PBS) with 2% carboxymethylcellulose (CMC, Sigma-Aldrich, USA) for oral infection.

2.2. Mice and treatments

Eight-week-old female BALB/c Cr Slc (BALB/c) mice (20–25 g), purchased from Vital River Inc. (Beijing, China), were divided randomly into three groups (n = 8 per group) under specific-pathogen-free conditions. The Institutional Animal Care and Use Committee of Peking University Health Science Center approved all the animal protocols (approval number LA2014242). The mice received sterile food and drinking water ad libitum, with (1) and (2) distilled water or (3) 0.02% solution of EGCG from 8 weeks to 15 weeks. EGCG was administered at a dose of 0.02% in drinking water following our previous study [14]. The EGCG derived from green tea (95% purity as determined by HPLC) was purchased from Sigma-Aldrich (Cat. no. E4143, USA) and dissolved in distilled water. Mice were orally inoculated at 2-day intervals, up to a total of 20 times, with (1) 100 μL PBS with 2% CMC or (2) and (3) 10^8 CFU of *P. gingivalis* in 100 μL PBS with 2% CMC, as described previously [17]. At the age of 15 weeks, the mice were sacrificed to collect blood, gingival tissue and maxillae samples.

2.3. Tissue collection and preparation

After the last injection, mice continued to receive food and distilled water with or without EGCG until 15 weeks, and then were euthanized by intraperitoneal pentobarbital sodium (100 μg/μL, Merck, Germany). Blood samples were collected by infraorbital puncture, and serum was isolated by centrifugation at 10,000 rpm for 5 min at 4 °C. The gingival tissue was rapidly removed from mice into a liquid N_2 box and kept at −80 °C until analyzed, or fixed by 10% formalin for immunohistochemical staining. Mice maxillae were harvested and fixed in 4% paraformaldehyde at 4 °C overnight, and then transferred to a 70% ethanol solution. Horizontal bone loss around the maxillary molars was scanned by Micro-CT (Inveon MM CT, Siemens, USA).

2.4. Micro-CT imaging

Micro-CT imaging of mice maxillae was performed using a Siemens Inveon MM CT scanner for the generation of three-dimensional model. Parameters were as follows: 360° rotation, 360 projections, 1500 ms exposure time, 60 kV source voltage, 220 μA beam current, and effective pixel size of 8.82 μm. Acquisitions were reconstructed with a filtered back projection algorithm, matrix size 1024 × 1024 × 448, using Inveon Acquisition Workplace software (Siemens, USA). Images were rotated and adjusted from M1 to M3, and then analyzed by Inveon Research Workplace software (Siemens, USA) to measure the distance from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) for evaluating the bone resorption.

2.5. Mouse inflammation cytokine antibody array

Serum samples were analyzed using the Mouse Inflammation Ab array C1 (RayBiotech, USA) consisting of 40 different inflammation cytokine antibodies, following the manufacturer's recommendation as previously described [18,19]. Signal intensities from the bound cytokines were measured using the equipment of Fusion FX Vilber Lourmat (Marne-la-Vallée cedex, France) and analyzed using mouse inflammation cytokine semi-quantitative software. Six spots of positive control were served by Biotin-conjugated IgG, where it was used to identify the membrane orientation and to normalize the results from different membranes which were being compared. The net optical density level from each spot was determined by subtracting the background optical level from the total raw optical density and the level of each cytokine was represented as a percentage of the positive control.

2.6. Cytokine ELISA

Serum samples were isolated from blood after euthanasia (15 weeks), and cytokine levels were detected using Enzyme-linked immunosorbent assay (ELISA) kits for IL-17 and IL-1β (RayBiotech, USA).

2.7. Immunohistochemical staining

Gingival tissues were fixed by 10% formalin and embedded in paraffin. The sections (5 μm thick) were processed by deparaffinized, rehydrated and heat-induced epitope retrieval. Hydrogen peroxide (3%) was used to block the endogenous peroxidase for 10 min at room temperature. Sections were blocked by goat serum and then incubated with rat anti-mouse IL-17 (Bioss, China) and rat anti-mouse IL-1β (Boster, China) as primary antibodies overnight at 4 °C. After washing with PBS three times, the secondary antibody (IgG) was dropped on at 37 °C for 20 min, and washed by PBS three times. Avidin biotin horseradish peroxidase (S-A/HRP) was then added to the slides for visualization, and they were stained by diaminobenzidine (DAB) for detection. Finally, nuclei were counterstained with Mayer's hematoxylin and mounted with permount (Sinopharm Chemical Reagent, China). For the observation of the positive staining area, images were acquired using an Olympus BX41 microscope (Olympus, Japan) at ×400 magnifications, and analyzed by using Image-Pro Plus software (American Media Cybernetics co, USA).

2.8. Quantitative real-time PCR

Total RNA was purified from gingival tissues using an RNeasy Plus Mini Kit (Qiagen, USA), and then reverse-transcribed using a Primescript RT Master Mix Kit (Takara Bio, Japan) to generate cDNA. Quantitative real-time PCR analysis was performed by using the Applied Biosystems 7500 Fast Real-time PCR System (Life Technologies, USA) in accordance with the manufacturer's protocol. Briefly, the reactions contained 10 μL of 2× SYBR Green (Takara Bio, Japan), each primer at 100 nM and 30 ng of reverse-transcribed RNA. The PCR conditions were as follows: 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 34 s. The dissociation curve analysis was then performed to confirm specificity. Each gene was tested in triplicate, and target RNA levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Primer sequences details are shown in Table 1.

Table 1
Primer sequences used for the real-time PCR.

Primers	Forward	Reverse
IL-1β	CTATACCTGTCCTGTGTAATGAAAGA	TCTGCTTGTGAGGTGCTGATGTA
IL-6	TAGCTACCTGGAGTACATGAAGAACA	TGGTCCTTAGCCACTCCTTCTG
IL-17	CCTCAGACTACCTCAACCGTTC	ACTGAGCTTCCCAGATCACAGAG
IL-23	GAGCAACTTCACACCTCCCTACTA	TGCCACTGCTGACTAGAACTCA
TNF-α	AGGCGGTGCCTATGTCTCAG	GCCATTTGGGAACTTCTCATC
CCL2	GCATCCACGTGTTGGCTCA	CTCCAGCCTACTCATTGGGATCA
RANKL	GCACACCTCACCATCAATGCT	ACCCTTAGTTTTCCGTTGCTTA
MMP-2	GATAACCTGGATGCCGTCGTG	CTTCACGCTCTTGAGACTTTGGTTC
MMP-9	CCCTGGAACTCACACGACATC	GTCCACCTGGTTCACCTCATG
GAPDH	AGGTTGTCTCCTGCGACTTCA	CTGTTGCTGTAGCCGTATTCATTG

2.9. Statistical analysis

All results are expressed as the mean ± standard deviation (SD). Data were analyzed using the two-tailed Student's test or one-way ANOVA, following Tukey–Kramer multiple tests by SPSS software (IBM). $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Micro-CT analysis for bone resorption

Alveolar bone resorption was analyzed by forming three dimensional structures using a Micro-CT scanner, which had a high sensitivity and accuracy for investigating and evaluating the bone loss in an animal model. Three dimensional reconstructions of left maxillae represented the palatal surface of the alveolar bone, and the length of bone loss from CEJ to ABC parameters indicated the difference among groups by displaying the six spots from M1 to M3. The distance of the alveolar bone process showed a higher bone loss in the *P. gingivalis*-challenged mice compared with the PBS-inoculated mice (Fig. 1A). Quantification of bone resorption presented a statistical increase in the *P. gingivalis*-challenged group compared with the PBS-inoculated group (Fig. 1B, PBS: 167 ± 16 mm vs. *P. gingivalis*: 196 ± 19 mm, $P < 0.05$). On administration of EGCG, the distance of the alveolar bone process was lower than in the *P. gingivalis*-challenged mice (Fig. 1A). Quantification data revealed a statistically-significant decrease in the EGCG treatment group (Fig. 1B, *P. gingivalis*: 196 ± 19 mm vs. *P. gingivalis* + EGCG: 150 ± 18 mm, $P < 0.01$).

3.2. Inflammatory mediator levels in serum

To analyze the levels of inflammation in serum between the different groups, mouse inflammation antibody array C1 was used to detect the intensities of 40 mouse inflammatory mediators. Semiquantitative membrane-based antibody arrays were subtracted and the data were normalized by positive control signals to analyze the inflammatory mediators (Fig. 2A). *P. gingivalis* challenge increased serum levels of the inflammatory cytokines IL-1β ($P < 0.05$), IL-6 ($P < 0.05$), IL-9 ($P < 0.01$), IL-12p70 ($P < 0.05$), IL-17 ($P < 0.05$) and TNF-α ($P < 0.05$), as well as other inflammatory mediators exotain-1 ($P < 0.05$), exotain-2 ($P < 0.05$), fas ligand ($P < 0.01$), monocyte chemoattractant protein (MCP)-1 ($P < 0.05$), monokine induced by IFN-γ (MIG) ($P < 0.05$) and macrophage inflammatory protein (MIP)-1α ($P < 0.05$), compared with the PBS-inoculated mice (Fig. 2B). The high expressions of inflammatory cytokines IL-1β ($P < 0.05$), IL-6 ($P < 0.05$), IL-9 ($P < 0.01$) and IL-12p70 ($P < 0.05$) were decreased by EGCG treatment, IL-17 and TNF-α were slightly decreased without being statistically significant. The increase in inflammatory mediators exotain-1 ($P < 0.05$), exotain-2 ($P < 0.05$), fas ligand ($P < 0.01$), MCP-1($P < 0.05$), MIG ($P < 0.01$), MIP-1α ($P < 0.01$) caused by *P. gingivalis* infection were also reduced in the EGCG-treated group (Fig. 2B).

3.3. Cytokine ELISA data in serum

Cytokine ELISA was performed to characterize the levels of inflammatory risk cytokines (IL-17 and IL-1β) in periodontal disease. Serum samples from the *P. gingivalis*-challenged mice expressed increased levels of inflammatory cytokines IL-17 (Fig. 3A; PBS: 1170.5 ± 97.9 pg/mL vs. *P. gingivalis*: 1265.9 ± 29 pg/mL, $P = 0.041$) and IL-1β (Fig. 3B; PBS: 814.5 ± 124.3 pg/mL vs. *P. gingivalis*: 976.6 ± 123.2 pg/mL, $P < 0.05$) compared with PBS-inoculated mice. In contrast, EGCG treatment decreased the level of IL-1β (Fig. 3B; *P. gingivalis*: 976.6 ± 123.2 pg/mL vs. *P. gingivalis* + EGCG: 531.6 ± 127 pg/mL,

(A)

PBS

P. gingivalis
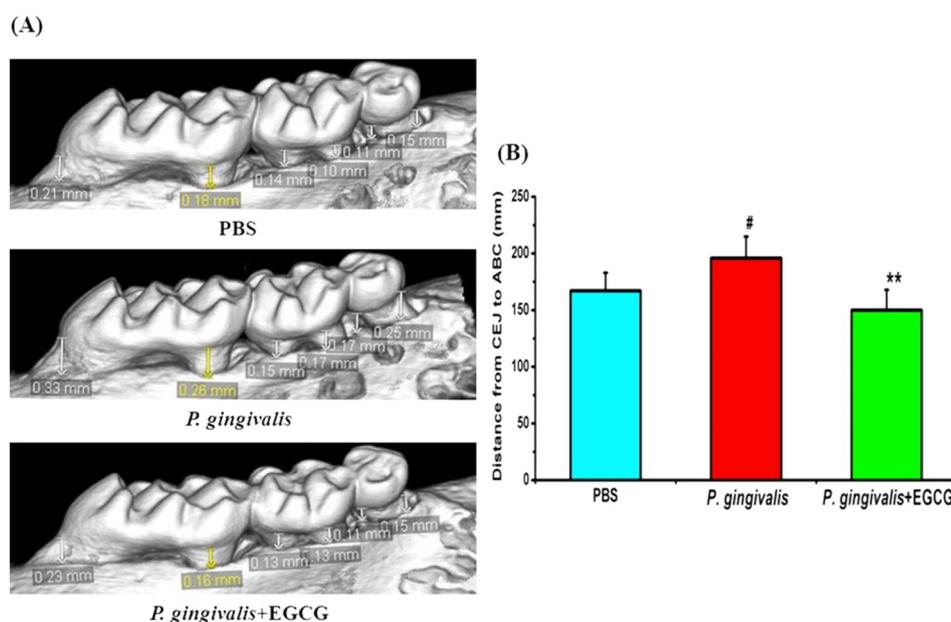
P. gingivalis+EGCG

(B)

Fig. 1. Alveolar bone resorption in mice was measured by micro-computed tomography. Oral infection with *P. gingivalis* elevated bone resorption in mice. Consumption of EGCG reduced alveolar bone loss caused by *P. gingivalis* infection (A). The Micro-CT analysis of distance from CEJ to ABC was shown at 15 weeks (B). Data are expressed as mean ± SD (n = 8). #$P < 0.05$ compared with the mice given PBS. **$P < 0.01$ compared with the *P. gingivalis*-challenged group.
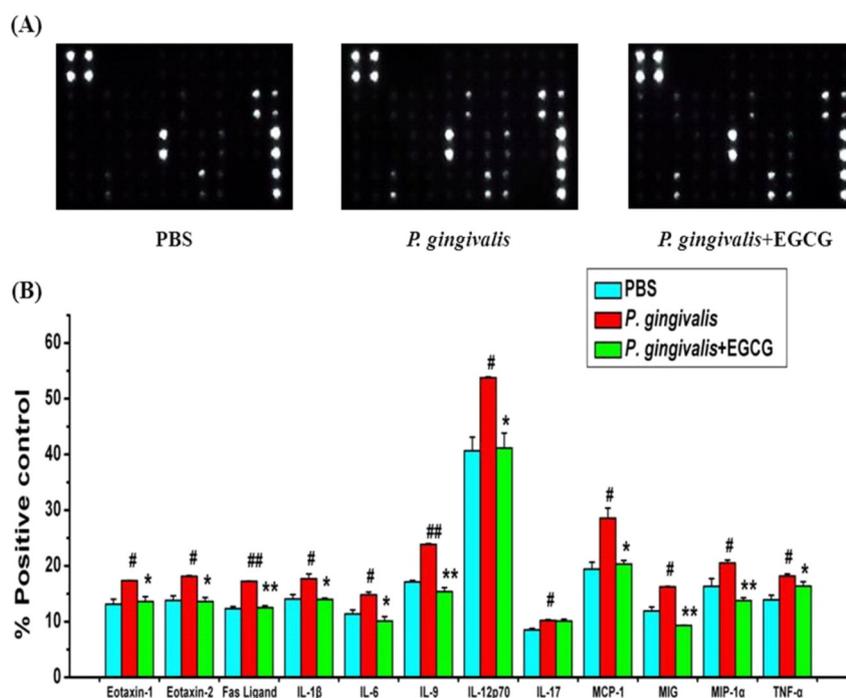
Fig. 2. Configuration of inflammatory mediators from *P. gingivalis*-challenged and EGCG-treated serum samples collected at 15 weeks (A). Percentage of the positive control evaluated the level of each cytokine (B). The data represent the mean ± SD from two independent experiments of each sample. #$P < 0.05$, ##$P < 0.01$ compared with the PBS-inoculated group.*$P < 0.05$, **$P < 0.01$ compared with the *P. gingivalis*-challenged group.

$P < 0.01$) obviously, but IL-17 level was not statistically significance (Fig. 3A; *P. gingivalis*: 1265.9 ± 29 pg/mL vs. *P. gingivalis* + EGCG: 1233.7 ± 147.2 pg/mL, $P > 0.05$).

3.4. Immunohistochemical analysis in gingival tissue

To investigate the inflammatory cytokines IL-17 and IL-1β from gingival tissue induced by *P. gingivalis* infection in BALB/c mice at 15 weeks, the sections of gingival tissue were stained by using IL-17 and IL-1β Abs. Immunohistochemical staining of the paraffin-embedded gingival tissue sections revealed positive histological changes in IL-17 and IL-1β production from *P. gingivalis*-challenged mice compared with PBS-inoculated mice. High positive expression of IL-17 and IL-1β areas in the gingival tissue were observed in the *P. gingivalis*-challenged mice. In contrast, administration of EGCG reduced the expression of IL-17 and IL-1β in gingival tissue by *P. gingivalis* infection (Fig. 4).

3.5. Real-time PCR detection of inflammatory mediators in gingival tissue

To examine the involvement of various inflammatory mediators in periodontitis activated by *P. gingivalis*, the level of gene expression in the gingival tissue from each group of mice was determined by real-time PCR. *P. gingivalis* induced an increase in mRNA expression of the main inflammatory risk cytokines IL-17 ($P < 0.05$), IL-23 ($P < 0.01$), IL-1β ($P < 0.05$), IL-6 ($P < 0.05$) and TNF-α ($P < 0.05$). Compared to the PBS-inoculated group, RANKL ($P < 0.01$) and CCL2 ($P < 0.01$) were also elevated by *P. gingivalis* infection, but there was no statistical significance between the PBS-inoculated group and the *P. gingivalis*-challenged group in MMP2 and MMP9. In contrast, the EGCG-treated group showed significantly decreased expression of IL-1β ($P < 0.01$), IL-6 ($P < 0.01$), TNF-α ($P < 0.05$), RANKL ($P < 0.01$), CCL2 ($P < 0.01$) and MMP-9 ($P < 0.05$) in gingival tissue, but not IL-23. The expression of IL-17 and MMP-2 were slightly down-regulated by EGCG treatment without being statistically significant (Fig. 5).

4. Discussion

Inflammation is the key factor for the initiation and development of periodontal disease associated with *P. gingivalis* infection, as *P. gingivalis* invades gingival tissue to cause an inflammatory response resulting in alveolar bone destruction. Many studies have shown that the oral pathogen infection by *P. gingivalis* leads to periodontal disease and bone resorption in both clinical investigations [4,20] and animal models, such as mice, rats and pigs [17,21,22].

Recently, more research is focused on natural products, which can regulate or prevent inflammatory diseases. Therefore, we investigated the potential of using EGCG's pharmacological functions for suppressing periodontitis caused by *P. gingivalis* infection. Firstly, EGCG can regulate inflammatory mediators, and may contribute to an anti-inflammatory effect for inhibiting bone resorption during *P. gingivalis* infection. The Mah and Jung research groups have reported that EGCG has anti-inflammatory effect in in vitro and in vivo studies [23,24].

We accelerated periodontitis in mice by oral infection with *P. gingivalis*, and then identified the anti-inflammatory effect of EGCG on periodontal disease. The alveolar bone process demonstrated a higher bone loss in *P. gingivalis*-challenged mice, which was attenuated by EGCG treatment by Micro-CT measurement. Nakamura et al. also reported that EGCG can inhibit LPS-induced bone resorption in vivo [25].

Inflammatory mediators include cytokines, chemokines and adhesion molecules associated with the recruitment of inflammatory cells, involving T-lymphocytes, monocyte/macrophages and neutrophils, which regulate cellular signaling for the expression of host inflammatory response. IL-6 and TNF-α, as key inflammatory cytokines in response to *P. gingivalis* infection, may be associated with periodontitis and regulation of bone metabolism. High expression of RANKL could enhance osteoclast formation and bone resorption [26,27]. IL-1β is an important cytokine for inducing RANKL or other mediators resulting in inflammatory response for bone loss. Furthermore, IL-1β mediates osteoclast differentiation and function by regulating the expressions of ERK 1/2, p38, p-JNK, MAPKs, c-Fos, and NFATc1 [28]. Recently, IL-17 worked on the osteoclastogenesis-supporting cell to induce RANKL and some inflammatory cytokines, such as IL-1β, IL-6 and TNF-α [29,30]. The correlation
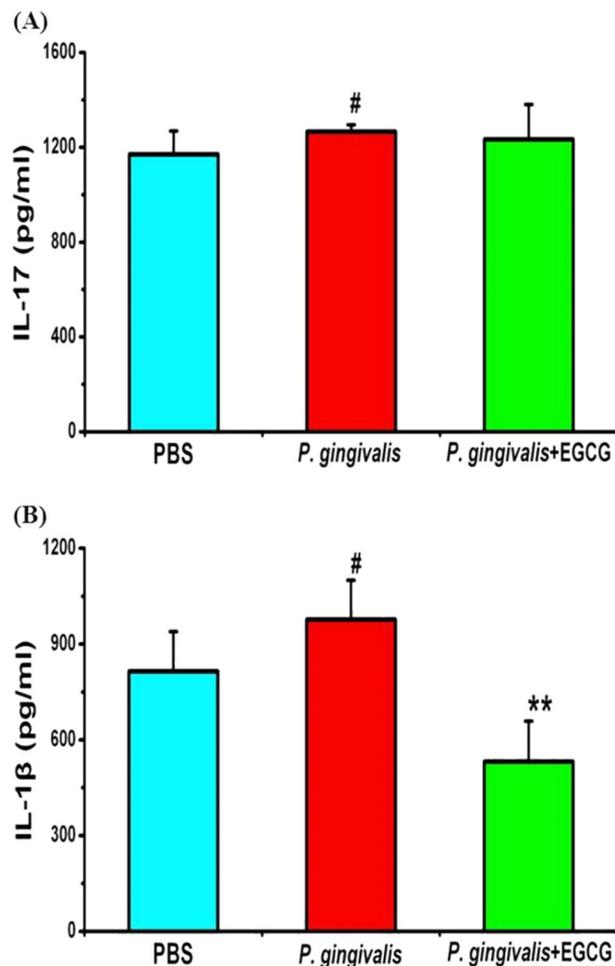
Fig. 3. Serum IL-17 and IL-1β levels of PBS-inoculated, *P. gingivalis*-challenged and EGCG-treated samples were gathered at 15 weeks (A and B). Data are expressed as the mean ± SD (n = 8). [#]$P < 0.05$ compared with the PBS-inoculated group. [**]$P < 0.01$ compared with the *P. gingivalis*-challenged group.

between Th17 cells and IL-1β protein demonstrate that IL-1β is a key promoter of Th17 cell differentiation, and it also showed that IL-1β primarily stimulates the expansion of Th17 cells and the expression of IL-17 [31,32]. Moreover, some reports showed IL-17 and IL-23 correlated with bone destruction, Th17 as a specialized inflammatory subset, has the potential for osteoclastogenesis to the bone loss [33,34]. IL-17 secreted by Th17 cells is not only important in autoimmune diseases, but also participates in local inflammatory processes in tissues.

In our study, those series of cytokines (IL-17, IL-1β, IL-6 and TNF-α), along with IL-9, IL-12p70, Eotaxin-1, Eotaxin-2, Fas ligand, MCP-1, MIG and MIP-1α were elevated by *P. gingivalis* infection, and decreased by EGCG treatment using a mouse inflammation antibody array. Furthermore, the expression of IL-17 and IL-1β in ELISA and immunohistochemical staining showed the increased in the *P. gingivalis*-challenged group, which was reduced by EGCG treatment. From real-time PCR data, the EGCG-treated group significantly lowered the expression of IL-1β, IL-6, TNF-α, RANKL and CCL2, which were elevated in *P. gingivalis*-challenged mice from gingival tissue except IL-23, and slightly down-regulated IL-17 and MMP-2. Although IL-17 is not significantly decreased, EGCG can down-regulate most inflammatory mediators in serum and gingival tissue as well as attenuate bone loss for osteoclast metabolism via RANKL variation by *P. gingivalis* infection.

It has been reported that EGCG suppress the activation of NF-κB which controls gene expression for most inflammatory mediators, such as cytokines, chemokines, adhesion molecules and MMPs [35]. Yang and Singh also demonstrated that EGCG can inhibit the pro-inflammatory cytokine levels of IL-1β, IL-6 and TNF-α in some cell lines and murine models [36,37]. EGCG suppress RANKL-induced activation of JNK/c-Jun and NF-κB pathways by anti-osteoclastogenic effect for reducing the expression of c-Fos and NFATc1 in osteoclast precursors, and it can prevent IL-1β induced bone resorption and osteoclast formation in vivo [38]. Only a few studies report that EGCG can inhibit the periodontitis induced by *P. gingivalis* infection in rodent models [26,39], so the research observations for this topic are still limited. Therefore, our study manifested that the anti-inflammatory effect of EGCG could inhibit inflammatory mediators to alleviate the periodontitis induced by *P. gingivalis*.

Our observation indicates that EGCG may be inhibiting bone resorption by down-regulating most inflammatory mediators without significant differences in IL-17. In contrast, Hosokawa et al. found that EGCG and ECG prevented IL-17A mediating CCL20 production in human gingival fibroblasts, providing direct benefits in periodontal disease by catechin flavonoids [40]. There are several possibilities as to why IL-17 is not significantly reduced by EGCG. We suspect that IL-17 as a pro-inflammatory cytokine, perhaps maintained a high expression during the initial period of the experiment, and could not be down-regulated by EGCG immediately after the last infection. Another possible reason is that the level of IL-23, which mediates the differentiation of Th17 cells and induces IL-17 production in mouse models, was not reduced by EGCG. Moreover, the expression of IL-23 in macrophages is related to inflammation and more IL-17-expressing T-cells in human periodontal lesions. Sato et al. clarified the role of the IL-17/IL-23 axis in bone metabolism by investigating IL-17 −/− and IL-23 −/− mice [33], and it also reported bone resorption appeared normal in deficient mice to indicate that neither IL-17 nor IL-23 can regulate the bone homeostasis respectively in vivo [34]. LPS-induced model of inflammatory bone destruction suggest that Th17 cells expanded through IL-23 stimulation are involved in the T cell mediated osteoclastogenesis [32]. Although IL-17 and IL-23 were not down-regulated by EGCG treatment, it has been demonstrated that oral administration of EGCG could inhibit bone loss in rats with experimental periodontitis in accordance with our study [26,39]. Moreover, perhaps the concentration of EGCG was too low to down-regulate the expression of IL-17, but we used this low concentration following most studies to prevent inflammatory diseases without any untoward effect. Mazzanti et al. have reported that consumption of too much green tea would lead to hepatotoxicity [41], and high doses of EGCG would promote inflammatory mediators.

Secondly, EGCG has an anti-bacterial effect against variety of organisms, and destroys cell membranes and cell walls of *P. gingivalis* [42]. Furthermore, consumption of EGCG disrupts the initial step in biofilm formation and inhibits the adherence of *P. gingivalis* by anti-bacterial effect [43]. The anti-biofilm effect of EGCG is transient, so we ensured continuous intake of EGCG to maintain a certain concentration level to prolong its activity in our study. Lastly, EGCG plays a role in protection against oxidative stress related inflammatory disease due to its anti-oxidative effect [44]. Because oxidative stress could damage the fibroblast cells and induce cell apoptosis. Moreover, *P. gingivalis* LPS and hypoxia increases oxidative stress in periodontal ligament fibroblasts and contributes to periodontitis [45].

In conclusion, our results demonstrate that inflammatory mediators play a key role in the development of periodontitis in BALB/c mice after *P. gingivalis* infection, which can be alleviated by EGCG treatment. Although IL-17 levels were not significantly decreased by EGCG treatment, it still indicates that oral continuous administration of EGCG is an effective method for down-regulating inflammatory response and attenuating bone resorption. Our findings provide a potential therapeutic effect for EGCG to prevent periodontal disease by down-regulating inflammation.

Conflict of interest
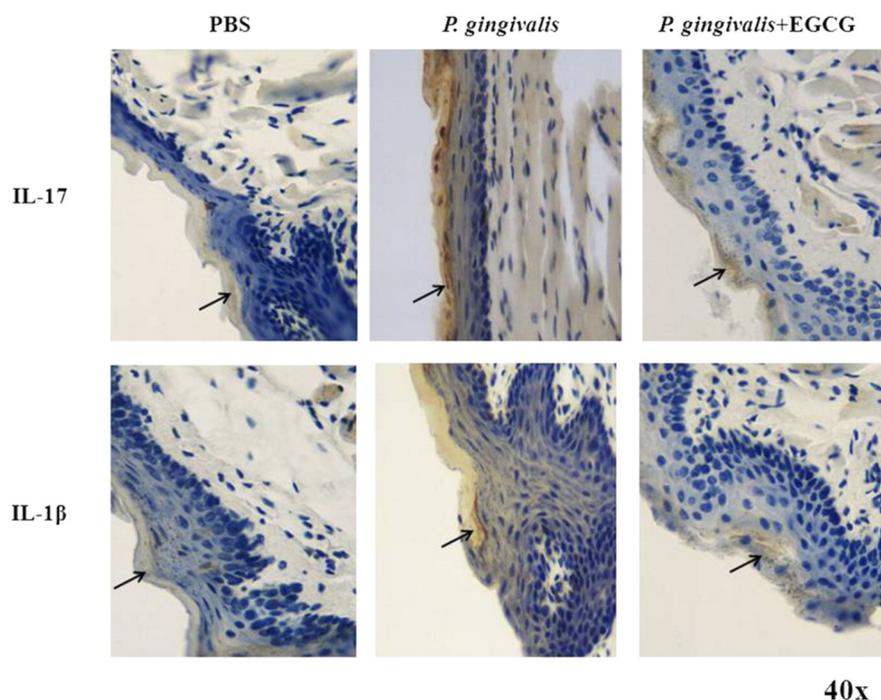
The authors report no conflicts of interest.

Fig. 4. Immunohistochemical analysis of IL-17 and IL-1β from the paraffin-embedded gingival tissue of BALB/c mice in PBS, *P. gingivalis*-challenged and EGCG-treated groups represented the positive histological changes. Arrows indicate the positive-stained area.

Author contribution statement

Y. C. and QX. L. designed and conducted the research. Y. C., ZB. C., H. L., and Y. X. provided essential reagents and materials. Y. C. and XX. W. analyzed the data. Y. C. and QX. L. wrote the paper. QX. L. had primary responsibility for the final content. All authors have reviewed the manuscript.
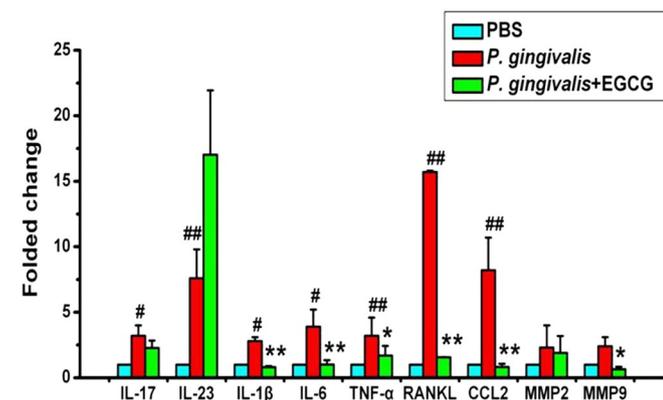
Acknowledgments

Fig. 5. The relative mRNA gene expression of inflammatory mediators was determined by real-time PCR in PBS-inoculated, *P. gingivalis*-challenged and EGCG-treated mice at 15 weeks. The levels of inflammatory mediators were obtained after normalization to GAPDH. Values represent the mean ± SD (n = 8). Data are expressed as the folded change increase in the mRNA level compared with the PBS-inoculated group (A), and decrease by EGCG-treatment (B). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the PBS-inoculated group.$^{*}P < 0.05$, $^{**}P < 0.01$ compared with the *P. gingivalis*-challenged group.

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